University of Alberta

Regulation of secretion of parathyroid hypertensive factor

by

Sharla Kae Sutherland ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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Sharla Kae Sutherland

Box 13, Site 2, R. R. 3
Barrhead, Alberta
Canada, T7N 1N4

January 28, 2003
Date submitted to FGSR

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Regulation of secretion of parathyroid hypertensive factor** submitted by ***Sharla Kae Sutherland*** in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Christina G. Benishin (Supervisor)

Johanne Tremblay (External examiner)

Loren W. Kline

Richard Z. Lewanczuk

Sandra T. Davidge

Susan Jacobs (Chairperson)

January 27, 2003
Date approved by committee

For mom, dad, Chris, and Marc; my family, my life.
Your support, love, and understanding are invaluable.

Abstract

Low-renin hypertension, a subset representing 30-40% of all essential hypertension, displays several abnormalities in the Ca^{2+}-regulating system including hypocalcemia, elevated 1,25-dihydroxyvitamin D3 ($1,25\text{-}(OH)_2D_3$), and increased parathyroid gland (PTG) activity. This increased PTG activity is manifested through elevated plasma parathyroid hormone (PTH) and the newly described parathyroid hypertensive factor (PHF), a substance which is believed to play a causal role in the pathogenesis of hypertension. The work presented here investigates the regulation of PHF secretion both directly, and indirectly through regulation of parathyroid cell proliferation, in the low-renin hypertensive rat model, spontaneously hypertensive rat (SHR) and the normotensive genetic control Wistar Kyoto (WKY) rats.

SHR parathyroid cells (PTC) were shown to proliferate at a faster rate, and grow to a greater plateau density than WKY PTC. As well, exposure to either low extracellular (EC) Ca^{2+} or $1,25\text{-}(OH)_2D_3$ was associated with increased PTC number. SHR PTC also secreted PHF to a greater extent than WKY PTC under basal conditions. Low EC Ca^{2+} rapidly and differentially stimulated PHF release depending on the rat strain, model used, and the density of the cell culture. The extent of stimulation may be partially explained by strain differences and cell density-related changes in calcium-sensing receptor (CaR) protein since the CaR was shown to play an important role in mediating the effects of EC Ca^{2+} on PHF release. $1,25\text{-}(OH)_2D_3$ also rapidly stimulated PHF release with enhanced sensitivity in SHR vs WKY cultures indicated by a leftward shift in the dose-response curve, whereas $24,25\text{-}(OH)_2D_3$ had the converse effect. As well, evidence suggested potential involvement of the plasma membrane-bound vitamin D receptor $1,25VDR_{mem}$,

which was increased in SHR versus WKY parathyroid cells in the mechanism of action of $1,25\text{-}(OH)_2D_3$.

These results support the idea that PTG hyperplasia exists in low-renin hypertension in rats and could indirectly contribute to elevated plasma PHF. Furthermore, these data also suggest that hypocalcemia and $1,25\text{-}(OH)_2D_3$ may contribute to elevated plasma PHF in the SHR.

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Abbreviations

$1,24,25\text{-}(OH)_3D_3$,	1,24,25-trihydroxyvitamin D3
$1,25\text{-}(OH)_2D_3$,	1,25-dihydroxyvitamin D3
$1,25VDR_{mem}$,	membrane VDR for $1,25\text{-}(OH)_2D_3$
$1,25VDR_{nuc}$,	nuclear VDR for $1,25\text{-}(OH)_2D_3$
1α–OHase,	25-hydroxyvitamin D3-1α-hydroxylase
$24,25\text{-}(OH)_2D_3$,	24,25-dihydroxyvitamin D3
$24,25VDR_{mem}$,	membrane VDR for $24,25\text{-}(OH)_2D_3$
$24,25VDR_{nuc}$,	nuclear VDR for $24,25\text{-}(OH)_2D_3$
24-OHase,	25-hydroxyvitamin D3-24-hydroxylase
25-OHase,	vitamin D3-25-hydroxylase
$25\text{-}OHD_3$,	25-hydroxyvitamin D3
AngII,	angiotensin II
ATP,	adenosine triphosphate
ATPase,	adenosine triphosphatase
AVP,	arginine vasopressin
B2,	monoclonal antibody directed against parathyroid hypertensive factor
BSA,	bovine serum albumin
BT,	$1,24(OH)_2\text{-}22\text{-ene-}24\text{-cyclopropyl-}D_3$
Ca^{2+},	ionized calcium
cAMP,	cyclic adenosine monophosphate
CaR,	calcium-sensing receptor
cDNA,	complementary deoxyribonucleic acid
CRF,	chronic renal failure
DAG,	diacylglycerol
Dahl-SR,	Dahl-salt resistant
Dahl-SS,	Dahl-salt sensitive
DBP,	vitamin D binding protein
DMEM,	Dulbecco's modified Eagle medium
DNA,	deoxyribonucleic acid
DOCA,	desoxycorticosterone acetate
DTT,	dithiothreitol
EC,	extracellular
ECL,	enhanced chemiluminescense

ECM,	extracellular matrix
EDTA,	ethylenediamine-tetraacetic acid
EGTA,	ethyleneglycol-bis(b-amino ethyl ether)N,N'-tetraacetic acid
ELISA,	enzyme-linked immunosorbant assay
FBS,	fetal bovine serum
FITC,	fluorescein isothiocyanate
HBSS,	Hank's balanced salt solution
HPLC,	high performance liquid chromatography
hPTH,	human PTH
IgM-3A,	polyclonal antibodies directed against parathyroid hypertensive factor
IP3,	inositol triphosphate
K$^+$,	ionized potassium
kDa,	kilodaltons
MEN,	multiple endocrine neoplasia
Mg^{2+},	ionized magnesium
mmHg,	millimetres mercury
mRNA,	messenger ribonucleic acid
Na$^+$,	ionized sodium
NE,	norepinephrine
PAGE,	polyacrylamide-gel-electrophoresis
PBS,	phosphate-buffered saline
PDE,	phosphodiesterase
PHF,	parathyroid hypertensive factor
PKA,	protein kinase A
PKC,	protein kinase C
PLC,	phospholipase C
PTC,	parathyroid cells
PTG,	parathyroid glands
PTH,	parathyroid hormone
PTHrP,	parathyroid hormone-related peptide
RAA,	renin-angiotensin-aldosterone
RNA,	ribonucleic acid
SDS,	sodium dodecyl-sulphate
SHR,	spontaneously hypertensive rats
TBST,	tris-buffered saline with tween-20
Tween,	polyaxyethylenesorbitan monolaurate
UV,	ultraviolet radiation
VDE,	vitamin D3 responsive element
VDR,	vitamin D receptor

VSMC,	vascular smooth muscle cells
WKY,	Wistar Kyoto rats

Chapter 1

Literature Review

1 LITERATURE REVIEW

1.1 Introduction

Calcium has many diverse functions in life-sustaining processes and thus the maintenance of adequate plasma concentrations of calcium is critical to an organism's survival. The parathyroid gland (PTG) monitors and regulates plasma calcium (and phosphate) through secretion of parathyroid hormone (PTH) which subsequently acts on the principal calcium-regulating tissues, bone, kidneys and intestine to bring about increases in plasma calcium. PTH exerts its effects in concert with 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), which itself is activated by PTH. A third calcium-regulating hormone, calcitonin, functions to suppress plasma calcium; although its role in calcium metabolism appears to be related to fine-tuning rather than overt control of plasma Ca^{2+} levels.

Until recently, the parathyroid glands were thought to only play a role in the physiology of calcium and phosphate homeostasis. However, with the observation of hypertension in a substantial proportion, but notably not all, of primary hyperparathyroid patients (1-8), a role for the PTG in the pathogenesis of hypertension was suspected. Several lines of evidence supported this assertion. First, parathyroidectomy of these patients often relieved hypertension (3, 8). Second, parathyroidectomy and parathyroid cross-transplantation of low-renin hypertensive rat models indicated that hypertension was linked to the PTG (9-11). In addition, plasma abnormalities indicative of hyperparathyroidism such as hypocalcemia and elevated PTH and 1,25-$(OH)_2D_3$, were observed in both low-renin rat models (12-15) and human hypertensives (16-18). Finally, spontaneously hypertensive rats (SHR) were shown to have parathyroid abnormalities such as increased mass and the presence of a "novel cell" type, the percentage of which was correlated with blood pressure (19).

These observations led to speculation regarding the role of the only known parathyroid product at the time, PTH, in hypertension. However examination of the work of Pang and others (20-23) clearly revealed that PTH was actually a hypotensive hormone and therefore was unlikely to be playing a role in the pathology of hypertension.

Concurrent with these studies were the suggestions that there was a circulating hypertensive factor in essential hypertension. Given that the PTG of low-renin hypertension was not only over-active, but a required element of hypertension, and that hypertension prevailed despite elevated PTH (which was hypotensive), the source of this hypertensive factor was postulated to be the PTG. Thus plasma derived from several low-renin hypertensive rat models and human hypertension was examined for pressor activity. The fraction of partially purified plasma in the 1-5 kDa range was indeed shown to induce a delayed rise in blood pressure that correlated with increased ionized calcium (Ca^{2+}) uptake and influx through L-type Ca^{2+} channels. The PTG were confirmed to be the source of this substance, thus it was named "parathyroid hypertensive factor" (PHF).

The trigger for enhanced PHF secretion in low-renin hypertension is unknown. Dietary salt loading has been shown to stimulate the appearance of PHF in plasma in salt-sensitive hypertension, a subset which is co-existent with low-renin hypertension. As well, dietary studies have shown that calcium supplementation can suppress plasma PHF. The direct effect of extracellular (EC) Ca^{2+} on PHF release has also shown that Ca^{2+} can inhibit PHF release. PHF was quantified in these studies by the blood pressure bioassay, however, recently an ELISA for PHF detection has been developed which has enabled more precise examination of the regulation of PHF release. The work presented here is focused on the regulation of PHF secretion and characterization of the parathyroid abnormalities in low-renin hypertension as they may ultimately relate to enhanced PHF secretory capacity. A greater knowledge of these processes will provide the basis for the investigation of potential therapeutics which may be used to treat low-renin hypertension.

The purpose of this literature review is to provide the reader with a background of the essential concepts regarding the PTG, hypertension, and PHF. This will be accomplished first through an overview of calcium metabolism and the roles of the calcium-regulating hormones. The physiology and pathophysiology of the PTG will then be reviewed along with an examination of the evidence linking hyperparathyroidism, hypocalcemia, calcium-regulating hormones, and the PTG with low-renin hypertension. Lastly, there will be a comprehensive discussion of the parathyroid hypertensive factor (PHF), followed by a summary and the presentation of a hypothesis and the aims of this thesis.

1.2 Calcium metabolism

Calcium is required for a wide variety of physiological processes. It functions in both extracellular and intracellular capacities to maintain appropriate conditions for life. The extracellular actions of Ca^{2+} include neurotransmission; both exocrine and endocrine hormone secretion; stimulus-contraction coupling; and fertilization (24, 25). Regulation of vascular tone and development of contractile force in muscle cells; signal transduction in response to extracellular cues; regulation of many enzyme activities; and regulation of cell adhesion molecules are among the diverse intracellular effects of Ca^{2+} (26, 27) In addition to the regulatory roles, calcium is a major structural component of the skeleton and teeth (28). Therefore homeostasis of plasma calcium is crucial for an organism's survival, thus a complex regulatory system has evolved for this purpose.

1.2.1 Overview

The principle calcium-regulating hormones are PTH; the biologically active vitamin D3 metabolite, 1,25-$(OH)_2D_3$; and calcitonin. PTH is synthesized and secreted from the PTG and ultimately functions to raise plasma calcium and lower plasma phosphate. 1,25-$(OH)_2D_3$ is synthesized through sequential hydroxylations, first at the liver then at the kidney, of the vitamin D3 precursor produced in the epidermis in response to ultraviolet (UV) irradiation. The actions of 1,25-$(OH)_2D_3$ are, like PTH, to raise plasma calcium, but unlike PTH, to also raise plasma phosphate. Calcitonin is synthesized and released from the C-cells of thyroid and lowers both plasma calcium and phosphate. These three hormones act at three major target sites to bring about changes in plasma calcium and phosphate. The intestines, a classical 1,25-$(OH)_2D_3$ target and newly discovered PTH target (29, 30), are the site of dietary calcium and phosphate absorption. PTH, 1,25-$(OH)_2D_3$, and calcitonin also regulate the bone processes of resorption and formation to provide a source of plasma calcium when intestinal absorption of calcium is insufficient. The kidneys are the final site of calcium and phosphate metabolism with regulation of reabsorption by all three calcium-regulating hormones. The PTG complete this endocrine axis and act as the master control center for calcium and phosphate metabolism by providing the site for negative feedback control of PTH release primarily by Ca^{2+}, phosphate, 1,25-$(OH)_2D_3$. For the sake of simplicity and the purposes of this thesis, the focus of the following sections will be on calcium metabolism (illustrated in Figure 1-1 on page 47). The regulation of phosphate homeostasis will not be reviewed.

1.2.2 Calcium-regulating hormones

1.2.2.1 Calcitonin

Calcitonin is a single chain 32 amino acid peptide and is one of two alternative splicing products of the initial mRNA transcript of the calcitonin gene (the other product being calcitonin gene-related peptide) (31, 32). Synthesis of calcitonin occurs in the C-cells of the thyroid (33, 34) and results from cleavage of a calcitonin precursor peptide (35, 36). Since the overall effect of calcitonin on plasma calcium is inhibitory, release of calcitonin from C-cells is regulated primarily by EC Ca^{2+} with high plasma Ca^{2+} stimulating release (37).

1.2.2.2 Vitamin D metabolites

Vitamin D is a secosteroid which is actually not a vitamin at all, but an endogenous prohormone. Vitamin D3 (cholecalciferol) is the naturally occurring form of vitamin D whereas vitamin D2 (ergocalciferol) is obtained only from plant sources. Synthesis begins in the skin with the formation of previtamin D3 from 7-dehydrocholesterol following UV exposure (38). Previtamin D3 is then converted to vitamin D by a temperature-dependent isomerization process. Vitamin D3 is then absorbed into the subepidermal microcirculation and transported in the plasma bound to the vitamin D binding protein (DBP) (39, 40). Conversion of vitamin D3 to its biologically active form occurs through two sequential hydroxylations in the liver and kidney (41). The first hydroxylation is by cytochrome P450 oxidase 27 (vitamin D3-25-hydroxylase; 25-OHase) of carbon 25 of vitamin D (42). 25-hydroxyvitamin D (25-OHD_3) is then transported to the kidney where the tightly regulated cytochrome P450 oxidase 1α (25-hydroxyvitamin D-1α-hydroxylase; 1α-OHase) catalyzes hydroxylation of carbon 1 of 25-OHD_3 yielding biologically active 1,25-$(OH)_2D_3$ (43). During conditions of adequate calcium and 1,25-$(OH)_2D_3$, 25-OHD_3 is hydroxylated at carbon 24 by cytochrome P450 oxidase 24 (25-hydroxyvitamin D-24-hydroxylase; 24-OHase) (39).

Although initial reports suggested that 24,25-$(OH)_2D_3$ may be an agonist for the 1,25VDR_{nuc} and can exert similar effects to 1,25-$(OH)_2D_3$ (44, 45), the role for 24,25-$(OH)_2D_3$ in calcium-metabolism specifically appears to be minor (46-50). However, effects of 24,25-$(OH)_2D_3$ in the process of bone formation/mineralization (51, 52) and skeletal development (53, 54) and inhibitory effects on bone resorption (55) have been demonstrated, and more recently, antagonist effects of 24,25-$(OH)_2D_3$ have been shown in the intestine for 1,25-$(OH)_2D_3$ and PTH-mediated Ca^{2+} absorption (56). 24-OHase is expressed nearly ubiquitously and will also inactivate the biologically active 1,25-$(OH)_2D_3$ to the inert 1,24,25-trihydroxyvitamin D3 (1,24,25-$(OH)_3D_3$) (39).

1.2.2.3 PTH and PTHrP

Parathyroid hormone is formed by the sequential enzymatic cleavage of a 115 amino acid prepro-PTH to the 90 amino acid pro-PTH, and finally to PTH (84 amino acids) in the PTG (57). Amino acid residues 1-6 are responsible for activation of adenylate cyclase through the PTH/PTHrP receptor whereas residues 7-34 are involved in receptor binding (57). The regulation of PTH synthesis and secretion will be discussed in detail in section 1.2.7. PTHrP is a 141 amino acid protein which shares amino acid identity with PTH for 8 of the first 13 amino acids and exerts its actions through the PTH/PTHrP receptor (58). PTHrP protein or mRNA is expressed in a variety of tissues and cell types including central nervous system (59), cardiovascular system (60, 61), pancreas (62), and keratinocytes (63). PTHrP was first discovered as a secreted product from a variety of malignant tumors (64-67) and was shown to be responsible for the hypercalcemia of malignancy (66); however, circulating levels of PTHrP are much lower than for PTH therefore PTHrP is not believed to be a physiological regulator of plasma Ca^{2+} (66, 67).

1.2.3 Receptors

PTH, vitamin D3 metabolites, and calcitonin all exert their effects through specific receptors which are differentially expressed to confer tissue-specific effects. A brief overview of the receptor sub-types and distribution for PTH, vitamin D3 metabolites, and calcitonin is useful prior to examining calcium metabolism at the major target tissues.

1.2.3.1 PTH Receptors

PTH exerts its calcium homeostatic functions through the PTH/PTHrP receptor-1. This monomeric G-protein-coupled receptor protein with 7 transmembrane domains is expressed in osteoblasts and the kidneys and is, as the name indicated, activated by both PTH and PTHrP (68-70). At least two different signaling pathways, adenylate cyclase and phospholipase C (71-73), are coupled to this receptor and are activated by distinct domains of the PTH molecule. Specifically, the first two amino acids of the N-terminus of PTH are required for activation of adenylate cyclase (74), whereas residues 25-34 are required for activation of the protein kinase C (PKC) pathway (75). Activation of this receptor in bone and kidney tissue results in rapid increases in inositol triphosphate (IP3) and diacylglycerol (DAG) levels (76) as well as intracellular Ca^{2+} (77, 78). The PTH/PTHrP receptor is also found in various non-classical target sites such as vascular smooth muscle (79) and keratinocytes (80). A second distinct receptor for PTH, PTH receptor type II or PTH2 has also been identified (81, 82) which is also coupled to both the AC and PLC signaling pathways (81, 83-85). This receptor is expressed in brain, pancreas, testis, placenta (82), as well as arterial and cardiac endothelium, vascular smooth muscle, lung (86), thyroid parafollicular cells (87) and possibly dermal fibroblasts (85), however is not expressed in the classical calcium-regulating tissues bone and kidney (85, 86). The physiological importance of this receptor is unclear although a role in the hypothalamus has been suggested (88). A third PTH receptor which is specific for the C-terminal region of PTH has also been described in osteoblast-like cells and rat parathyroid cells (89)

1.2.3.2 Vitamin D Receptors

The previous dogma for the mechanism of action of $1,25\text{-}(OH)_2D_3$ reflected the classical steroid modality of signaling via an intracellular receptor with either cytosolic or nuclear distribution. Such a receptor for $1,25\text{-}(OH)_2D_3$ ($1,25VDR_{nuc}$) was discovered in 1969 in the chromosomal material within nuclei of chick intestinal enterocytes (90) and subsequently cloned from rat kidney (91), and chick (92) and human (93) intestinal tissue. This receptor belongs to the steroid hormone receptor superfamily (94) and was ultimately shown to reside largely in the nucleus where, when occupied, it functions as a transcription factor (95). For upregulatory signaling, occupation of the $1,25VDR_{nuc}$ with $1,25\text{-}(OH)_2D_3$ leads to formation of heterodimer signaling complexes with the retinoid X receptor followed by complex binding with upstream gene regulatory sequences of DNA (vitamin D3 responsive element, VDE) and association of appropriate accessory and regulatory proteins (95, 96). In the PTG, downregulatory signaling such as inhibition of PTH transcription occurs in a similar manner with the exception that the retinoid X receptor does not appear to be involved (97). The tissue distribution of the $1,25VDR_{nuc}$ extends far beyond the classical target sites involved in calcium and phosphate metabolism as nearly every tissue is represented (98) revealing the vast array of physiological functions of $1,25\text{-}(OH)_2D_3$.

Accompanying this wide range of functions was the observation that $1,25\text{-}(OH)_2D_3$ could exert rapid effects in a variety of tissues in a manner analogous to peptide-based signaling. For example, $1,25\text{-}(OH)_2D_3$ has been shown to induce rises in intracellular Ca^{2+} and stimulate phospholipid metabolism in enterocytes (99), osteoblasts (100), and parathyroid cells (101, 102). This discovery prompted the search for a rapid signal transduction mechanism. A novel cell plasma membrane-bound $1,25\text{-}(OH)_2D_3$ receptor, $1,25VDR_{mem}$, was identified and found to mediate a variety of rapid effects including stimulation of PKC (103) and antiproliferative effects of $1,25\text{-}(OH)_2D_3$ on chondrocytes (104). The $1,25VDR_{mem}$ is also expressed in intestinal epithelia (105), chick kidney and brain (106) and differentiated osteoblasts (107). The ligand specificities of the 1,25VDR$_{mem}$ are different from that of the classical 1,25VDR$_{nuc}$ (108, 109) suggesting that the proteins are physically distinct. 1,25-$(OH)_2D_3$ is composed of an eight carbon side chain, a seco B-ring, and the A-ring all of which may occupy different positions resulting in numerous 1,25-$(OH)_2D_3$ conformers. The unique seco-steroid nature of 1,25-$(OH)_2D_3$ allows for high conformational flexibility and rotation about the 6-7 single carbon bond yielding either 6-s-cis or 6-s-trans B-ring conformers, as well as chair-chair interconversions of the A-ring (110). The binding requirements for the 1,25VDR$_{mem}$ include a 6-s-cis locked planar conformation, and either a side chain C-20 "S" or "R" conformation of 1,25-$(OH)_2D_3$ (110, 111). In contrast, binding of 1,25-$(OH)_2D_3$ specifically to the 1,25VDR$_{nuc}$ requires a B-ring orientation with either an α or β configuration, semi-rigid side chain, and a preferential side chain C-20S conformation (110).

Receptors for the important, but less studied, vitamin D3 metabolite, 24,25-$(OH)_2D_3$, have also been identified. Nuclear uptake of 24,25-$(OH)_2D_3$ in cartilage (112) and high affinity binding of 24,25-$(OH)_2D_3$ to a macromolecule in cytosolic and nuclear fractions of long bone tissue of newborn rats (53) and in differentiating skeletal mesenchyme (113) suggest the presence of a specific nuclear receptor for 24,25-$(OH)_2D_3$. Whether this receptor is unique from the classical 1,25VDR$_{nuc}$ and whether 1,25-$(OH)_2D_3$ can compete for binding is unknown, however the high binding affinity reported in one study suggests that 24,25-$(OH)_2D_3$ binds a specific and unique receptor (53). The presence of a 24,25-$(OH)_2D_3$ receptor in the PTG has also been suggested (114). As for 1,25-$(OH)_2D_3$, rapid effects of 24,25-$(OH)_2D_3$ have been observed suggesting the presence of a cell plasma membrane-bound receptor for 24,25-$(OH)_2D_3$. Such a receptor was indeed identified and is thought to mediate the rapid effects of 24,25-$(OH)_2D_3$ observed in some tissues such as rapid inhibition of 1,25-$(OH)_2D_3$ and PTH-induced Ca^{2+} transport across intestinal epithelia (56). This receptor was shown to be distinct from the $1{,}25VDR_{mem}$ (107) and was demonstrated in chondrocytes (104), osteoblasts (107), and enterocytes (115). As described in section 1.2.2.2, 24,25-$(OH)_2D_3$ appears to play only a minor role in calcium metabolism and non-calcium-regulating roles have only been described for the cardiovascular system (see section 1.3.2.7.2).

1.2.3.3 Calcitonin Receptors

The calcitonin receptor was cloned near the same time as the PTH/PTHrP receptor and, like the PTH/PTHrP receptor, was also shown to be a G-protein coupled receptor with 7 transmembrane domains. This receptor is coupled to both the adenylate cyclase and the phospholipase C (PLC) signaling pathways (116, 117) and is expressed in the classical calcitonin target sites, bone and kidneys (118, 119).

1.2.4 Role of bone in Ca^{2+} homeostasis

The immediately available pool of Ca^{2+} in bone is the first line of defense against hypocalcemia and provides for acutely required adjustments in plasma calcium. Bone can also respond to regulation by PTH, 1,25-$(OH)_2D_3$, and calcitonin to provide for more chronic adjustments in plasma calcium through the processes of resorption and formation, liberating and sequestering calcium respectively. The reversible process of calcium incorporation into the hydroxyapatite crystal lattice of bone is mediated through the activities of osteoblasts (bone builders) and osteoclasts (bone resorbers). The formation of bone as part of routine remodeling occurs through deposition of extracellular matrix

(ECM) molecules secreted by the osteoblast. Bone resorption is facilitated by the macrophage-like osteoclasts. These specialized cells extend pseudopodia, adhere to the bone lattice, and secrete acid into this isolated compartment resulting in bone resorption.

Both 1,25-$(OH)_2D_3$ and PTH are required for *in vivo* skeletal resorption as either one alone is incapable of fulfilling this process (120). The predominant effect of PTH on bone is stimulation of bone resorption, although anabolic effects on bone are also observed after acute exposure to low doses of PTH. PTH regulates bone activity largely through osteoblasts and expression of the PTH/PTHrP receptor protein is greatest in the less differentiated preosteoblasts (121). Since Ca^{2+} liberation is achieved through the activity of bone-resorbing osteoclasts, it is odd that receptors for PTH are not present on osteoclasts. It is believed that the bone-resorbing effects of PTH are therefore indirect and communicated from osteoblasts to osteoclasts (122), possibly through release of a secreted factor (123).

The critical role of 1,25-$(OH)_2D_3$ in normal bone physiology was established with the observation that rickets and osteomalacia induced by vitamin D deficiency could be reversed with 1,25-$(OH)_2D_3$ treatment (40). It was thus thought that 1,25-$(OH)_2D_3$ was primarily a bone-building hormone. This is supported by observations that 1,25-$(OH)_2D_3$ stimulates synthesis of Type I collagen and alkaline phosphatase activity (necessary for bone mineralization and matrix maturation) in mature osteoblasts, although not in proliferating osteoblast precursors (124-126). Other bone-building associated proteins are also stimulated by 1,25-$(OH)_2D_3$ including osteopontin (127), fibronectin (128), and osteocalcin (129). However, this bone-building activity does not seem to be in keeping with the classical role of 1,25-$(OH)_2D_3$ as a calcemic hormone and in fact, long-term exposure to 1,25-$(OH)_2D_3$ was shown to result in bone resorption (130). Similar to PTH, 1,25-$(OH)_2D_3$ also regulates bone-resorbing activity largely through osteoblasts (122), which signal to osteoclasts possibly through a secreted factor (131). As well, 1,25-$(OH)_2D_3$ stimulates transport of Ca^{2+} from the bone fluid compartment to the plasma (132, 133). Unlike PTH however, 1,25-$(OH)_2D_3$ may also contribute to bone resorption by stimulating differentiation of osteoclast precursor cells into a mature osteoclast phenotype (134).

The classical physiological effect of calcitonin is suppression of plasma calcium therefore the effect of calcitonin on bone is inhibition of bone resorption. This is accomplished through direct inhibition of osteoclast function (135, 136).

1.2.5 Role of the intestines in Ca^{2+} homeostasis

The primary calcium-regulating functions of the intestines are to absorb dietary calcium used for skeletal formation and growth, and in adulthood, to counteract the calcium lost through kidneys and urination. The high surface area of the brush-border membrane of the intestines allows for maximal calcium and phosphate reabsorption. The major route for calcium transport across the epithelial barrier is first through Ca^{2+} influx via Ca^{2+} channels followed by association with Ca^{2+}-binding proteins (calbindins) and transcellular movement within endocytic vesicles or lysosomes (137) along microtubule networks (138, 139).

1,25-$(OH)_2D_3$ plays an important role in calcium absorption. The classical, genomic functions of 1,25-$(OH)_2D_3$ include enhanced gene transcription of calbindins (140, 141) and increased microtubule protein expression (142). 1,25-$(OH)_2D_3$ has also been shown to alter intestinal brush border membrane topography to bring about an increase in surface area and therefore absorption (143), and stimulate vesicular transport of Ca^{2+} (138). More recently, rapid non-genomic effects of 1,25-$(OH)_2D_3$ have been demonstrated including stimulation of luminal Ca^{2+} absorption (144) through Ca^{2+} channel opening (145). Finally, 1,25-$(OH)_2D_3$ appears to downregulate intestinal expression of the $1,25VDR_{mem}$ protein, the receptor which is thought to mediate the rapid effects of 1,25-$(OH)_2D_3$ on Ca^{2+} transport (146).

The role of 24,25-$(OH)_2D_3$ is less clear. One report has shown that 24,25-$(OH)_2D_3$ can antagonize Ca^{2+} transport (56) stimulated by 1,25-$(OH)_2D_3$ and PTH in the perfused chick intestine. The inhibitory effects of 24,25-$(OH)_2D_3$ on 1,25-$(OH)_2D_3$- and PTH- induced stimulation of Ca^{2+} transport may be mediated by a suppression in the activities of PKC, protein kinase A (PKA), or both (56).

The intestines have historically not been a target for PTH. However, direct effects of PTH on Ca^{2+} uptake by isolated intestinal cells were first demonstrated by Nemere et al in 1981 (29, 147). Subsequent studies indicated that PTH rapidly stimulates Ca^{2+} transport across perfused, isolated, chick duodenal loops (30). The mechanism of action of PTH appears to occur through stimulation of both voltage-dependent Ca^{2+} channel currents (148) and adenylate cyclase (149), followed by downstream activation of mitogen-activated protein kinase (150). These results suggest a role for PTH in Ca^{2+} absorption in the intestines.

1.2.6 Role of the kidneys in Ca^{2+} homeostasis

The primary calcium-regulating function of the kidneys is to reabsorb most

(~99%) of filtered Ca^{2+} in the proximal tubule of the nephron, and fine-tune Ca^{2+} reabsorption according to physiological need through regulation in the distal tubule of the nephron. Reabsorption of filtered Ca^{2+} occurs in a similar manner as Ca^{2+} absorption in the intestine. Following entry through Ca^{2+} channels or endocytosis, Ca^{2+} binds calbindins, is transported across the cell, and absorbed across the basolateral membrane through Ca^{2+}-ATPase (adenosine triphosphatase) and Na^{+}/Ca^{2+} exchanger activities. Some Ca^{2+} may also be reabsorbed through the small spaces between tubular cells.

The site of regulation of these processes by PTH and 1,25-$(OH)_2D_3$ is the distal tubule of the nephron. PTH and 1,25-$(OH)_2D_3$ both stimulate Ca^{2+} reabsorption through activation of basolateral Ca^{2+}/Na^{+} exchangers (39, 40, 151). 1,25-$(OH)_2D_3$, like in the intestines, stimulates renal expression of calbindins (152, 153). Furthermore, as mentioned above, PTH also facilitates Ca^{2+} retention and absorption by activating the circulating precursor of bioactive vitamin D3, 25-OHD_3, through stimulation of renal 1-OHase (154-156). The activity of 1-OHase is inhibited, in classic product inhibition fashion, by 1,25-$(OH)_2D_3$ (155, 156). Conversely, activity of the vitamin D3 inactivating enzyme, 24-OHase, is inhibited by PTH and stimulated by 1,25-$(OH)_2D_3$ and Ca^{2+} (156, 157).

1.2.7 Parathyroid Glands

The critical role of the PTG in maintenance of plasma calcium was discovered when high mortality rates due to hypocalcemia were observed in a substantial proportion of post-operative thyroidectomy patients (158). In these patients, parathyroid tissue was inadvertantly removed during thyroidectomy due to the localization of a pair of parathyroid glands on each lobe of the thyroid. It was then realized that the parathyroid glands were vital for life.

The unique calcium homeostatic property of the PTG lies in their ability to sense and respond to changes in plasma Ca^{2+} with secretion of the calciotropic hormone, PTH. The plasma Ca^{2+} setpoint in normal humans is approximately 1.2 mM (159). There is a clear inverse sigmoidal relationship between plasma ionized calcium and PTH release (160); that is, small deviations in plasma calcium result in rapid PTH secretion responses from the PTG. This is not surprising since both hypo- and hyper- calcemia have life-threatening consequences.

1.2.7.1 Regulation by EC Ca^{2+}

The major regulator of PTH synthesis and/or secretion from the PTG is EC Ca^{2+}. Hypocalcemia has been shown to stimulate both PTH synthesis (161, 162) and secretion (163-166). The mechanism of action of EC Ca^{2+} involves the recently cloned G-protein-coupled calcium-sensing receptor (CaR) (167) and has been reviewed elsewhere (168). The pivotal role of EC Ca^{2+} in regulation of the PTG is demonstrated by parathyroid pathologies in which the plasma ionized calcium level is abnormal. Inactivating (familial hypercalcemia hypercalciuria and neonatal severe hyperparathyroidism (169) and the CaR mouse knockout model (170)) or activating (autosomal dominant hypocalcemia (171)) mutations in the CaR and corresponding hyper- or hypo- secretion of PTH provide strong evidence for the importance of the CaR in regulation of plasma Ca^{2+}.

EC Ca^{2+} regulates both PTH release through a variety of signal transduction pathways. Exposure to high EC Ca^{2+} results in activation of PLC and accumulation of

DAG and IP3 (172). Classical signaling dogma states that this would be followed by receptor-operated release of Ca^{2+} from intracellular stores, and indeed EC Ca^{2+} can induce elevations in cytosolic Ca^{2+} (173), and activation of PKC. Since high EC Ca^{2+} inhibits PTH release and stimulates accumulation of IP3 and DAG, PKC might be expected to mediate this inhibition of PTH release. However, under high EC Ca^{2+} conditions, PKC actually *stimulates* PTH release (174, 175) in part by suppressing the EC Ca^{2+}-induced rise in intracellular Ca^{2+} (176). These unusual observations may be resolved, at least partially, by the observation that DAG stimulates production of sphingosine which inhibits PKC activity in parathyroid cells (177). In a simplified view then, EC Ca^{2+} may induce rises in IP3, intracellular Ca^{2+}, and DAG; DAG in turn stimulates production of sphingosine which inhibits PKC which ultimately leads to reduced PTH release. Other signaling pathways are likely involved in regulation of PTH release, such as adenylate cyclase and PKA (178), although the role in EC Ca^{2+}-regulated PTH release is not clear.

Normally, as with many endocrine tissues, parathyroid tissue grows at a very slow rate (179). For example, the calculated parathyroid cell birth rate in young rats was reported as 53.2% of the total parathyroid cell number/year (180). However, under certain conditions, parathyroid cell proliferation can be induced; usually as a response to pathological disruptions in Ca^{2+} and phosphate metabolism. Hypocalcemia, for example, can stimulate an increase in the rate of parathyroid cell (PTC) proliferation (180-183) through a mechanism which appears to involve the calcium-sensing receptor (CaR) (184).

1.2.7.2 Regulation by vitamin D metabolites

The importance of the biologically active metabolite, 1,25-$(OH)_2D_3$, in regulation of PTH synthesis and secretion is second only to EC Ca^{2+}. 1,25-$(OH)_2D_3$ transcriptionally suppresses PTH synthesis, inhibits PTH release, and inhibits parathyroid cell proliferation (185). As mentioned above, the PTG contain the classical nuclear receptor for 1,25-$(OH)_2D_3$, 1,25VDR_{nuc} (186, 187) which is upregulated by its ligand (188). Both genomic and non-genomic effects of 1,25-$(OH)_2D_3$ have been described. The rapid effects of 1,25-$(OH)_2D_3$ include increased cytosolic Ca^{2+} (101) and phospholipid metabolism (189) although it is not known whether these effects are directly correlated to effects on PTH secretion. The rise in intracellular Ca^{2+} in parathyroid cells appeared to be specific to 1,25-$(OH)_2D_3$ as neither 25-OHD_3 nor 24,25-$(OH)_2D_3$ had significant effects. The Ca^{2+} channel blockers diltiazem and verapamil were also without effect suggesting that Ca^{2+} channels were not voltage-gated, but perhaps receptor operated leaving open the possibility for involvement of the 1,25VDR_{mem}. The classical genomic effects are mediated through 1,25-$(OH)_2D_3$ association with the 1,25VDR_{nuc}, followed by binding with a unique down-regulatory DRE (185) upstream to the PTH gene and association of appropriate accessory and regulatory proteins. In this fashion, the occupied receptor acts as a transcription factor to elicit suppression of PTH gene transcription.

As described above for EC Ca^{2+}, 1,25-$(OH)_2D_3$ also appears to play an important role in the regulation of parathyroid cell proliferation. Anti-proliferative effects of 1,25-$(OH)_2D_3$ have clearly been demonstrated *in vitro* (190). Not surprisingly then, vitamin D3 deficiency has been associated with parathyroid cell hyperplasia and is believed to play an important role in the pathogenesis of secondary hyperparathyroidism (described below).

Few studies have addressed the potential role of 24,25-$(OH)_2D_3$ in regulation of the PTG. In parathyroid tissue derived from primary and secondary hyperparathyroid patients (discussed in section 1.3.1), both 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ inhibited isoproterenol- and sodium fluoride- stimulated adenylate cyclase activity (44). As mentioned above, the existence of a nuclear 24,25-$(OH)_2D_3$ receptor in parathyroid tissue has been suggested (114). Some studies have shown that supraphysiological concentrations of 24,25-$(OH)_2D_3$ have minor inhibitory effects on PTH release in rat and bovine parathyroid cells (47, 191), however subsequent reports failed to show any effect of 24,25-$(OH)_2D_3$ on PTH release from normal or hyperplastic canine and bovine (48, 192), rat (192), and human (193) parathyroid tissue, even at relatively high doses. As well, indirect effects of 24,25-$(OH)_2D_3$ on PTH activity *in vivo* and direct effects on plasma PTH levels could not be demonstrated in rats (49, 192)

1.2.7.3 Regulation by Phosphate

Dietary studies with phosphorus also revealed that high phosphate intake could stimulate PTH secretion under physiological conditions (180). Elevated plasma phosphate as a consequence of chronic renal failure has also been shown to enhance PTH release *in vivo* (183). The stimulatory effects of phosphate on the PTG were initially explained by phosphate binding of ionized calcium thus reducing plasma Ca^{2+} and indirectly affecting the PTG. Indirect effects were also believed to occur through high phosphate-mediated reductions in 1,25-$(OH)_2D_3$ synthesis, thus resulting in the diminution of a potent PTG inhibitor. However, subsequent *in vitro* studies where EC Ca^{2+} was adjusted to a constant for various concentrations of phosphate revealed that phosphate exerted direct (i.e. independent from changes in EC Ca^{2+} or 1,25-$(OH)_2D_3$) stimulatory effects on both PTH release (194) and parathyroid cell proliferation (195) in parathyroid cell cultures. The mechanism of action of phosphate is unclear, but the existence of a specific phosphate receptor similar to other ion-sensing receptors (such as the CaR) has been postulated (196). It is now believed that dietary phosphorus restriction can play a significant role in ameliorating the symptoms of secondary hyperparathyroidism (discussed below).

1.2.7.4 Other substances secreted from PTG

Only a few substances have been described which are released from the PTG. These include the ubiquitous endocrine products, the chromogranins (earlier referred to as secretory product-I) and various fragments thereof, and endothelins. These substances appear to play a predominantly paracrine/autocrine role in the regulation of PTG secretory activity. The secretion characteristics of these substances and metabolism by the PTG will be briefly reviewed.

1.2.7.4.1 Chromogranin

Chromogranin A is colocalized with the secretory products of a variety of endocrine tissues, including PTH in the PTG. Previously known as secretory protein-I (197), chromogranin A was shown to be, like PTH, negatively regulated by EC Ca^{2+} (198-200). Unlike for PTH, 1,25-$(OH)_2D_3$ has been shown to stimulate chromogranin A secretion from cultured parathyroid cells (201) and following administration to normal rats (202). The only known function of parathyroid-derived chromogranin A appears to be inhibition of both PTH and its own release in a paracrine/autocrine fashion (198-200).

In addition, chromogranin A is a prohormone for several smaller peptides including pancreastatin (198) and betagranin (203). Both pancreastatin (198, 199, 204) and betagranin (203) act in a paracrine/autocrine manner to inhibit both chromogranin A and PTH synthesis and release from parathyroid cells. The physiological significance of the parathyroid production of chromogranin A and related peptides is not clear, but initial studies seem to indicate that they are involved in mediating and "fine-tuning" the effects of EC Ca^{2+} and possibly 1,25-$(OH)_2D_3$ on PTG activity.

1.2.7.4.2 Endothelins

Immunoreactivity for endothelin (ET)-1 protein and the presence of mRNA for ET-1 have indicated that the PTG synthesize this substance (205). It is believed to behave as an autocrine factor as highly specific receptors for ET-1 (ETA and ETB) have been detected on parathyroid cells (205, 206). An autocrine/paracrine role for endothelin-1 would be consistent with the observation that it appears to be degraded in the medium or taken up by parathyroid cells (205). A physiological role for ET-1 in modulating the regulation of PTH synthesis/secretion by EC Ca^{2+} has been suggested based on the responsiveness of endothelin-1 mRNA levels to ambient EC Ca^{2+} (205), and in fact, ET-1 was shown to stimulate PTH release (206). Regulation of PTH by ET-1 may be mediated by changes in intracellular Ca^{2+} and IP3 in parathyroid cells, although there are differential effects of ET-1 on cytosolic Ca^{2+} depending on exposure time (206). Thus the role of ET-1 in parathyroid cells appears to be somewhat complex. It is possible that ET-1 modulates the sensitivity of the parathyroid PTH response to EC Ca^{2+} (206).

1.3 Association between Hyperparathyroidism and Hypertension

1.3.1 Hyperparathyroidism

Hyperparathyroidism is defined by elevated plasma PTH as a result of PTG overactivity (primary hyperparathyroidism) or excessive parathyroid responses to chronically suppressed plasma Ca^{2+} and 1,25-$(OH)_2D_3$ (secondary hyperparathyroidism). The causes and contributors to hyperparathyroidism are varied and explanations are more readily available for secondary than for primary hyperparathyroidism.

Secondary hyperparathyroidism most commonly is the result of complications related to chronic renal failure, such as hypocalcemia and vitamin D3 deficiency. Reports of downregulated CaR (184) and 1,25VDR_{nuc} (207, 208) also suggest reduced inhibition of PTH synthesis and secretion at the site of the PTG. Alterations in the expression of these receptor proteins are likely secondary to the changes in plasma components accompanying chronic renal failure. Oncogene mutations have also been described in the hyperplastic parathyroid cells of secondary hyperparathyroidism (209). All of these complications would clearly aggravate the PTG overactivity resulting in even greater secretion of PTH than would be predicted based on the plasma profiles of Ca^{2+} and 1,25-$(OH)_2D_3$ alone.

In primary hyperparathyroidism, the causes are more elusive, and, as the name implies, related to some defect originating in the PTG. In primary hyperparathyroidism, as for secondary hyperparathyroidism, parathyroid cell hyperplasia results in increased maximal PTG secretory capacity and thus contributes to increased plasma PTH. Most diagnoses of primary hyperparathyroidism are of benign parathyroid cell hyperplasia or adenoma, alone, or in association with multiple endocrine neoplasia (MEN). Abnormalities in cell cycle regulation in some parathyroid adenomas have been reported (for review, see (210)) although no clear cause of primary hyperparathyroidism has been identified. PTG of primary hyperparathyroidism also have a higher set-point for suppression of PTH release by plasma Ca^{2+} which was correlated with reduced expression of the CaR (211, 212) suggesting that both protein levels of the CaR and post-CaR signaling are abnormal in primary hyperparathyroidism. As well, variations in the $1{,}25VDR_{nuc}$ gene have been linked to decreased $1{,}25VDR_{nuc}$ mRNA in primary hyperparathyroid patients (213).

Therefore although the PTG of both forms of hyperparathyroidism are hyperplastic and secreting excessive amounts of PTH, the distinction between primary and secondary hyperparathyroidism depends on whether this PTG response is appropriate (secondary hyperparathyroidism) or inappropriate (primary hyperparathyroidism) in the face of altered plasma Ca^{2+}, phosphate, and $1{,}25\text{-}(OH)_2D_3$ concentrations.

In addition to the obvious disruptions in Ca^{2+} and phosphate metabolism present in hyperparathyroidism, these patients are also frequently hypertensive. Depending on the study examined, a significant proportion (18-78%) of primary hyperparathyroid patients are also hypertensive (1-8). The cause of hypertension in these patients may be related to hypercalcemia, however this would not explain why not all primary hyperparathyroid patients are hypertensive. Parathyroidectomy has been shown to alleviate hypertension in some cases (3), but exacerbate in others (214). This has lead to substantial investigation of the role of the PTG, PTH, and calcium metabolism in hypertension. Similarly, a significant proportion of essential hypertensives exhibit features of secondary hyperparathyroidism. This relationship is explored in the proceeding sections.

1.3.2 Hypertension

1.3.2.1 Blood pressure

Blood pressure regulation is critical to survival of an organism and thus involves the input of many regulatory networks throughout the body. The maintenance of adequate blood pressure is necessary in order to have adequate tissue blood perfusion, and therefore adequate oxygen and nutrient delivery to and waste removal from tissues. Blood pressure is directly related to cardiac output (which itself is a function of heart rate and stroke volume) and inversely related to total peripheral resistance. Thus regulation of blood pressure may occur at a wide variety of target sites aimed at maintaining appropriate extracellular fluid volume, heart contractility and rate, and diameter of resistance vessels (arterioles). Blood pressure changes are detected by sensitive baroreceptors in the carotid and aortic arteries and transmitted to the vasomotor centre in the medulla via sympathetic sensory fibres. Increased firing of these fibres in response to an increase in blood pressure results ultimately in decreased sympathetic activity resulting in an overall decrease in blood pressure.

1.3.2.2 Hypertension—diagnosis, prevalence, and research

Hypertension, diagnosed when blood pressure exceeds either 140 mmHg systolic pressure or 90 mmHg diastolic pressure or both, is the result of a complex combination of genetic and dietary factors. Although itself mostly benign (except in the case of aneurysm), hypertension contributes to both cardiovascular disease and stroke (215), an association which is thought to be at least partially related to the excessive stress placed upon the vasculature and the pathological responses to this stress. Approximately one quarter of the North American population are hypertensive (216), however in only about 5% of cases can hypertension be attributed to an identifiable cause. The remaining 95% are termed "essential hypertensives".

In an attempt to reveal the etiology of essential hypertension, several animal models of hypertension, both genetic and experimentally induced, have been developed. Use of these animal models and studies of hypertensive humans have lead to the conclusion that hypertension is a condition with heterogeneous etiologies and may result from metabolic, endocrine, and/or other cellular abnormalities. An extensive analysis of the causal components of hypertension is beyond the scope of this thesis. However a discussion of the relationship between hyperparathyroidism and hypertension is particularly relevant and significant insight into the pathophysiology of hypertension has been gained by categorization of the renin profile.

1.3.2.3 The Spontaneously Hypertensive Rat

The spontaneously hypertensive rat (SHR) is the most studied rat model of hypertension (217). It was developed by Okamoto and Aoki in 1963 through inbreeding of normotensive Wistar Kyoto (WKY) rats (217). SHR first become hypertensive at approximately 5-6 weeks of age and attain a systolic blood pressure usually in the range of 180-200 mmHg. The survival of these animals varies between 10-21 months and death is related to the end-organ damage which is often, but not always (217), associated with hypertension. Complications exhibited by SHR include cardiac hypertrophy (218), cardiac failure (at 18-24 months of age) (219), kidney dysfunction indicated by proteinuria and decreased creatinine clearance (220, 221), and impaired endothelium-dependent relaxation (222). The SHR model however does not exhibit other signs of vascular dysfunction such as stroke, macroscopic atherosclerosis, and vascular thrombosis (217). The SHR also displays abnormalities at the cellular level. Hamet et al reported increased apoptosis in a variety of tissues including heart, brain, vascular smooth muscle and kidney cells (223) as well as increased vascular smooth muscle proliferation *in vitro* (224). Anti-hypertensive effects have been observed following treatment with angiotensin-converting enzyme (ACE) inhibitors (225, 226) or Ca^{2+} channel antagonists (226, 227). The complications of cardiac hypertrophy (225, 228), cardiac failure (229, 230), and endothelium impairment (231) have all been improved with both ACE inhibitor and Ca^{2+} channel blocker treatment. The pathophysiological abnormalities of the SHR associated with Ca^{2+} metabolism are discussed in subsequent sections.

1.3.2.4 Disturbances in calcium metabolism in essential hypertension

Disturbances in plasma ionized calcium and the calcium-regulating hormones in essential hypertension have been known for many years. It has been reported that patients with essential hypertension have an increased risk for developing hyperparathyroidism (1). As well, in rat models such as the SHR, suppressed plasma ionized calcium and elevated PTH have been observed (12, 13). Other abnormalities in SHR related to calcium homeostasis include renal calcium leak (232-234), decreased bone density and calcium content (235), and disturbances in intestinal calcium absorption

(236). Alterations in intestinal calcium absorption seem to depend on the age of the SHR, with pre-hypertensive young SHR exhibiting increased Ca2+ uptake in association with increased plasma 1,25-$(OH)_2D_3$ (237), whereas adult SHR appear to exhibit decreased Ca2+ absorption (235, 238). These findings all suggest a role for calcium and the calcium-regulating system in hypertension.

1.3.2.5 Renin profiling in essential hypertension

Renin profiling has resulted in significant insights into the heterogeneity of essential hypertension; in particular, the relationship between hyperparathyroidism and hypertension, as different renin subsets are associated with altered Ca^{2+} metabolism and corresponding shifts in the Ca^{2+}-regulating hormones (16). Plasma renin activity provides an index for the extent of activation of the renin-angiotensin-aldosterone (RAA) system (239), a system which has both direct vasopressor activities on the vasculature and indirect pressor activities due to volume retention effects.

The renin profile breakdown of essential hypertensives is approximately 30-40% low-renin, 20% normal renin, and 30-40% high-renin (239). The high renin hypertensives are hypercalcemic and thus have suppressed PTH and 1,25-$(OH)_2D_3$ and elevated calcitonin. Hypertension in this subset is believed to result largely from hyperactivation of the RAA system. The suspected cause(s) of hypertension in the normal-renin proportion is unknown.

Low-renin hypertension is the subset of interest as it exhibits the defects in calcium metabolism indicative of secondary hyperparathyroidism. This subset is characterized by both intracellular and extracellular abnormalities and is often associated with salt-sensitivity (i.e. dietary salt induces a pressor response) (17, 240). The intracellular abnormalities include elevated Ca^{2+} and Na^{+}, along with decreased intracellular Mg^{2+}, and pH (241-243). Alterations in the intracellular concentrations of these ions ultimately results in elevated intracellular Ca^{2+} which contributes directly to vascular smooth muscle contraction and thus vascular tone.

The plasma (extracellular) abnormalities of low-renin hypertension include suppressed plasma ionized calcium, and the anticipated shifts in the calcium-regulating hormones of increased 1,25-$(OH)_2D_3$ and PTH (12) and reduced calcitonin versus normotension (16, 17). Thus low-renin hypertension exhibits features of secondary hyperparathyroidism and therefore prompts examination of the roles of calcium and the calcium-regulating hormones in cardiovascular physiology and pathophysiology.

1.3.2.6 Role of calcium in low-renin hypertension

Dietary calcium studies in essential hypertension have yielded inconsistent results with some suggesting that calcium supplementation can ameliorate hypertension (244-246) and others showing no effect (247-249), or even a hypertensive effect (for review, see (250)). However, when plasma renin profile is taken into account, a high calcium diet has been shown to ameliorate hypertension in both low-renin rat models (251-254) and human hypertension (17, 255-257). Furthermore, only the subset of hypertensives with elevated PTH responded to dietary calcium supplementation with a reduction in blood pressure (258). In addition, salt-sensitive hypertension has also been shown to improve with dietary calcium loading (259). These studies are somewhat surprising given that hypercalcemia due to Ca^{2+} infusion can directly raise blood pressure under physiological conditions (i.e. in normotension) (260), partially through increasing Ca^{2+} availability to vascular smooth muscle. Concurrent with the anti-hypertensive effects of calcium-loading is the observation that Ca^{2+} channel blockers also have anti-hypertensive effects in low-renin hypertension (254, 261). This is a paradox since one treatment raises plasma Ca^{2+}, yet the other prevents its entry into cells. This paradox may be resolved with examination of the parathyroid gland's role in hypertension (discussed below) and further discussion of the roles of the calcium-regulating hormones (vitamin D3 metabolites and PTH) in the cardiovascular system and in hypertension.

1.3.2.7 Role of vitamin D3 metabolites in hypertension

1.3.2.7.1 1,25-$(OH)_2D_3$

Although 1,25-$(OH)_2D_3$ is classically known for its role in Ca^{2+} and phosphate homeostasis, a significant role has been defined in the cardiovascular system. Effects of 1,25-$(OH)_2D_3$ have been demonstrated on both cardiac and vascular smooth muscle. 1,25-$(OH)_2D_3$ has been shown to stimulate voltage-sensitive Ca^{2+} channel currents in classical vitamin D3 target sites, for example, intestines (145), therefore it is not surprising to find that 1,25-$(OH)_2D_3$ acts as a Ca^{2+} channel agonist in other tissues. Acute stimulation of L-type Ca^{2+} channel currents and increased intracellular Ca^{2+} (262) in vascular smooth muscle cells and positive inotropic actions in cardiomyocytes (263) are thus some of the important cardiovascular effects of 1,25-$(OH)_2D_3$ which could lead to increased blood pressure. As may be predicted then, 1,25-$(OH)_2D_3$ has been shown to enhance vascular contractility in response to classical vasopressors (264).

The overt effects of 1,25-$(OH)_2D_3$ on blood pressure are more complicated to discern. In some studies, bolus administration of $1,25\text{-}(OH)_2D_3$ in normotensive rats has not been shown to enhance blood pressure directly (i.e. independently from increases in plasma Ca^{2+}) after 24 hours but has been shown to augment the pressor responses to norepinephrine (NE) and angiotensin II (AngII) in vessels *ex vivo* (265) and increase vascular resistance in renal vascular beds *in vivo* (266). However in other studies, chronic administration of $1,25\text{-}(OH)_2D_3$ in normotensive rats resulted in a significant increase in blood pressure first apparent after 7 days of daily $1,25\text{-}(OH)_2D_3$ administration in the absence of any changes in plasma calcium (267). The effect of $1,25\text{-}(OH)_2D_3$ on blood pressure at more acute time points was also examined in these studies, however, consistent with human data (268), $1,25\text{-}(OH)_2D_3$ was not shown to have any early effects.

A chronic effect of $1,25\text{-}(OH)_2D_3$ in blood pressure regulation suggests several possibilities. A genomic mechanism of action of $1,25\text{-}(OH)_2D_3$ could account for the time lag and in fact, $1,25\text{-}(OH)_2D_3$ has been shown to increase synthesis of myosin light chain subunits (proteins which are involved in vascular smooth muscle contraction) in selected vascular beds (269). Alternatively, overt blood pressure effects of $1,25\text{-}(OH)_2D_3$ may not be detected because $1,25\text{-}(OH)_2D_3$ plays more of a modulatory role and influences the contractile responses of vascular smooth muscle to other more potent regulators.

Altered plasma concentration of and cardiovascular reactivity to $1,25\text{-}(OH)_2D_3$ have suggested a role for $1,25\text{-}(OH)_2D_3$ in experimental and human hypertension. Plasma $1,25\text{-}(OH)_2D_3$ has been shown to be elevated in low-renin and salt-sensitive rat models (14, 15, 270) and human hypertension (18). In some rat models, for example Dahl-salt sensitive (Dahl-SS) rats, this elevation in $1,25\text{-}(OH)_2D_3$ actually precedes the development of hypertension (15). In SHR however, elevated 1,25-$(OH)_2D_3$ appears to exist only in the young animal (237, 271) as there appears to be an age-related decline in 1,25-$(OH)_2D_3$ (272). In addition to altered plasma 1,25-$(OH)_2D_3$, chronic 1,25-$(OH)_2D_3$ administration has been shown to potentiate induced contractility *in vitro* in resistance vessels taken from SHR (264, 267, 273, 274) to a greater extent than for vessels derived from normotensive Wistar Kyoto (WKY) rats. As in normotension, studies of the effect of 1,25-$(OH)_2D_3$ on blood pressure have yielded inconsistent results. Transient effects on systolic blood pressure in male hypertensive patients (but not in normotensive volunteers) have been reported (268). In SHR, one report showed that sub-acute (7 day) treatment with 1,25-$(OH)_2D_3$ did not result in any significant effect on blood pressure (274), however, chronic administration (5-9 weeks) has been shown to accelerate the development of hypertension in the SHR (275). Although the studies of human hypertension yield a slightly different picture, the findings in SHR imply that 1,25-$(OH)_2D_3$ may exacerbate some pathway or predisposition to hypertension and thus contribute to the development of hypertension in the young SHR, but have no significant role in the maintenance of hypertension in the adult animal.

1.3.2.7.2 24,25-$(OH)_2D_3$

In contrast to 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ has been shown to exert rapid inhibition of both tonic and phasic vascular contraction induced by KCl, NE, or arginine vasopressin (AVP) treatment (276). These effects are observed in conjunction with inhibition of L-type Ca^{2+} currents in vascular smooth muscle cells (276). Of particular interest is the observation that 24,25-$(OH)_2D_3$ levels diminish during the progression of salt-sensitive hypertension in Dahl-SS rats leaving open the possibility that reduced

$24,25\text{-}(OH)_2D_3$-dependent inhibition of vascular contractility may contribute to salt-sensitive hypertension (277). *In vivo* however, administration of $24,25\text{-}(OH)_2D_3$ for two weeks in normotensive rats did not have direct hypotensive effects, as might be predicted, nor did it reduce the blood pressure and vascular responses to AngII and NE (265). Therefore the importance of $24,25\text{-}(OH)_2D_3$ in blood pressure modulation is not clear.

1.3.2.8 Unifying hypothesis for essential hypertension

Resnick et al have developed a hypothesis which generally accounts for the discrepancies observed in essential hypertension with respect to calcium metabolism (278, 279). All essential hypertension may be said to result from raised intracellular Ca^{2+} in vascular smooth muscle tissue. The cause of this rise in cytosolic Ca^{2+} is what distinguishes the low-renin and high-renin forms of hypertension. In low-renin hypertension, characterized by hypocalcemia and elevated PTH and $1,25\text{-}(OH)_2D_3$, hypertension would result from increased Ca^{2+} uptake from the extracellular space through the actions of $1,25\text{-}(OH)_2D_3$ and possibly other calcium-responsive hormones. The efficacy of therapeutic maneuvers such as increasing dietary calcium and Ca^{2+} channel blockers could then at least partially be explained through their ability to suppress plasma $1,25\text{-}(OH)_2D_3$ and prevent Ca^{2+} influx into vascular smooth muscle tissue. The role of dietary salt in the pathogenesis of low-renin hypertension is unclear, but it is known salt loading also results in elevated intracellular Ca^{2+}. Elevated plasma Na^+ may also have indirect effects on intracellular Ca^{2+} content by stimulating $1,25\text{-}(OH)_2D_3$ production (262).

In contrast, high-renin hypertension involves increased Ca^{2+} release from intracellular stores in response to agonists, such as AngII, which are elevated in the plasma. In these cases, increasing dietary calcium and Ca^{2+} channel blockers would have no beneficial anti-hypertensive actions.

In summary, Na^{+}- and Ca^{2+}-regulating hormones transduce environmental signals, such as dietary calcium and salt intake, at the level of vascular smooth muscle cells and the cellular responses to these hormones may be altered genetically in essential hypertension (280).

1.3.3 Involvement of PTG in low-renin hypertension

In addition to the association of hyperparathyroidism with low-renin hypertension, there are several lines of evidence which implicate the involvement of the PTG in hypertension. Parathyroidectomy in adult (9-11) and young (281) rat models of low-renin hypertension have resulted in a reduction of blood pressure. In human primary hyperparathyroid hypertensive patients however, parathyroidectomy resulted a decrease in blood pressure in some patients, but an increase or no effect in others (282). The diversity in responses to parathyroidectomy in these patients may be related to removal of both hypertensive and anti-hypertensive substances and will be discussed in subsequent sections. Furthermore, parathyroid cross-transplantation (9, 283, 284) studies have indicated that there is a linkage between hypertension and the PTG. These findings suggest that the PTG may be required for the pathology of low-renin hypertension. The association of hypertension with a subset of patients with primary hyperparathyroidism resulted in substantial speculation about the involvement of the PTG, and it's only known product at the time, PTH, in the pathogenesis or maintenance of low-renin hypertension.

1.3.3.1 Parathyroid abnormalities in low-renin hypertension

Somewhat surprisingly in light of the evidence indicating an association of the PTG with low-renin hypertension, parathyroid cell biology or regulation thereof in hypertension has been studied infrequently. Rather, the focus shifted to examination of the role of PTH in hypertension (discussed below). In one of the few published studies, SHR parathyroid tissue was shown to have an increased wet weight versus the normotensive genetic control strain, Wistar Kyoto (WKY) rats (285). The study indicated that the increase in mass was due to hyperplasia rather than gland hypertrophy, although the glands were not examined histologically. This same study also reported enhanced maximal specific [^{3}H]-1,25-$(OH)_2D_3$ binding in SHR parathyroid cell extracts. This is somewhat surprising since the SHR shares several features of secondary hyperparathyroidism due to chronic renal insufficiency in which reduced $1{,}25VDR_{nuc}$ expression has been reported (286). One explanation is that this enhanced maximal 1,25-$(OH)_2D_3$ parathyroid binding in the SHR may represent binding to the $1{,}25VDR_{mem}$ which has unknown parathyroid expression levels. Alternatively, the downregulation of the $1{,}25VDR_{nuc}$ in secondary hyperparathyroidism due to chronic renal failure may be an important part of the pathology of this condition in light of the drastically reduced plasma 1,25-$(OH)_2D_3$ levels and anti-proliferative effect of 1,25-$(OH)_2D_3$ in the PTG. In contrast, 1,25-$(OH)_2D_3$ is actually elevated in low-renin hypertension suggesting that hyperplasia is not related to insufficient 1,25-$(OH)_2D_3$.

A histological study of the SHR PTG showed that it possessed a novel cell type (19) shown in Figure 1-2. This cell type stained more darkly using hemotoxylin/eosin, carbohydrate, and lipid staining techniques. The cells were also highly vacuolated and the nuclei were pyknotic versus the surrounding normal parathyroid epithelial cells. The observation of these cells could not simply reflect an artifact due to tissue processing or staining procedures since novel cells represented approximately 15% of SHR PTG but were absent in glands derived from WKY rats. This implies that this cell phenotype may play a role in hypertension. This was supported by the observation of a correlation of the percentage of novel cells with blood pressure. These results suggest that these cells may either secrete or activate a substance which plays an important role in blood pressure regulation. Since PTH is elevated in low-renin hypertension, it is logical to examine the role of PTH in blood pressure regulation.

1.3.3.2 Cardiovascular effects of PTH

The hypotensive actions of a parathyroid extract were first demonstrated by Collip and Clark in 1925 (287). These effects were investigated in greater detail in studies by Charbon (23), Schleiffer et al (288), and Lindner et al (289). The most recent studies, spanning the last twenty years, have yielded an extensive body of evidence documenting the cardiovascular effects of PTH (20-22). The *in vivo* effects of PTH have been obscured by the ability of PTH administration to result in the plasma augmentation of two pressor elements, plasma 1,25-$(OH)_2D_3$ and Ca^{2+}. Therefore there has been at least one report of chronic PTH administration resulting in blood pressure-raising effects (290). None-the-less, most studies have shown a hypotensive effect of PTH. However, pharmacological doses are often required for the pressor effect of PTH which may indicate that secondary effects on 1,25-$(OH)_2D_3$ and ionized Ca^{2+} levels are occurring and interfering with the depressor actions of PTH. Alternatively, the requirement for supraphysiological concentrations of PTH may indicate that PTHrP (which can accumulate to relatively higher local concentrations), is actually the native ligand since comparative studies between PTH and PTHrP revealed that not only was PTHrP capable of exerting these effects, but that it exhibited substantially greater potency (291).

The specific hypotensive properties of PTH have been described in a variety of species and for many vascular beds (20-23). The mechanism of this inhibition appears to be related, at least in part, to the inhibition of L-type Ca^{2+} currents in vascular smooth muscle cells (292, 293). In contrast, in cardiomyocytes, L-type Ca^{2+} currents are stimulated by PTH which accounts for the positive inotropic and chronotropic effects of PTH (294, 295). It is believed that PTH exerts its effects on L-type Ca^{2+} channels through cyclic adenosine monophosphate (cAMP) signaling (296). The cardiovascular effects of PTH appear to have different peptide structural requirements from those of the classical Ca^{2+}-regulating effects (297). The calcemic effects of PTH are mostly confined to the first two N-terminal amino acid residues. However, the cardiovascular effects of PTH appear to be localized to amino acids 25-27 as native human PTH (hPTH(1-34)), but not alanine substituted PTH ([Ala25,26,27]hPTH(1-34)) was shown to be bioactive in relaxing precontracted rat tail arteries, inducing hypotension, and inhibiting the KCl-induced rise in intracellular Ca^{2+} in vascular smooth muscle cells (VSMC) in rats (298). These studies clearly indicate that the hypotensive properties of PTH can be attributed to distinct regions of the molecule separate from the region responsible for the hypercalcemic actions of PTH. Therefore, it is highly unlikely that PTH is contributing to the hypertension described in primary hyperparathyroidism and in fact, PTH has been shown to have hypotensive actions in primary hyperparathyroid patients (299).

1.4 Parathyroid Hypertensive Factor

1.4.1 Discovery of PHF

Parabiotic experiments whereby the circulation of a hypertensive rat was made continuous with the circulation of a normotensive rat had previously suggested the existence of a circulating hypertensive factor (300). With the PTG implicated in hypertension, many groups attributed hypertensive actions to PTH. However, as described above, the work of Pang and others has clearly demonstrated the hypotensive nature of PTH (20-22, 244). Since PTH is elevated in low-renin hypertension, the existence of a circulating antagonist of the hypotensive actions of PTH was suggested. In primary hyperparathyroid patients with hypertension, plasma ultrafiltrate was shown to raise platelet cytosolic Ca^{2+} *in vitro*; however, exposure to plasma processed from normotensive uremic patients with secondary hyperparathyroidism had no effect, despite equivalent levels of plasma PTH (10). These studies suggested the presence of a unique pressor molecule derived from or activated by the PTG in low-renin hypertension and hypertension of hyperparathyroidism.

In an attempt to detect and characterize this hypertensive factor, Lewanczuk and Pang injected partially purified plasma from SHR and normotensive WKY rats into normotensive assay animals and measured the blood pressure response over several hours. SHR, but not WKY plasma was shown not only to inhibit the hypotensive actions of PTH in the blood pressure bioassay (301), but to exert delayed hypertensive actions with peak activity 45-60 minutes after bolus injection (302). Small vasopressors and vasoactive ions which typically exert rapid pressor effects (within minutes) were removed by dialysis at a 1 kDa molecular weight cut-off and thus were not responsible for this delayed hypertensive action of SHR plasma extract. Therefore a circulating hypertensive factor in the plasma of SHR was proposed.

1.4.2 Purification and source of PHF

Because the PTG was associated with hypertension, the origin of the putative hypertensive factor was suspected to be the PTG. A 1-5 kDa fraction purified from culture medium of parathyroid gland and cell cultures was found to exert the classic delayed pressor response in the blood pressure bioassay suggesting that the major source of this hypertensive factor was indeed the PTG (303, 304), prompting the authors to term it "parathyroid hypertensive factor (PHF)" (305). PHF was subsequently shown to be sensitive to degradation by the enzymes trypsin, chymotrypsin, phospholipase C, and phospholipase D suggesting the presence of both a peptide and lipid component to PHF (306). The exact structure of PHF has yet to be determined although a partial peptide sequence has been obtained (see section 1.4.5).

Additional groups were also able to isolate substances of a similar molecular weight from the parathyroid tissue derived from human hyperparathyroid patients (10, 307) and from SHR plasma (308, 309) that share some of the unique structural and hypertensive features of PHF. One hypertensive substance in particular has been shown to be elevated in the plasma of SHR, however structural features such as a molecular mass of approximately 1 kDa, a hydrophilic nature, heat resistance, and insensitivity to trypsin or carboxypeptidase, suggest that the described substance is not PHF (310). This substance did not appear to be acting through the classical vasoconstriction pathways as antagonists to receptors for AngII, NE, thromboxane, or serotonin did not abolish the pressor effect of the substance (310).

The parathyroid origin of PHF was suggested by the absence of PHF activity following parathyroidectomy of hypertensive primary hyperparathyroid patients (10, 282) and cross-transplantation studies between SHR and normotensive rat strains (9). Bioactivity and biochemical purification properties (such as high performance liquid chromatography (HPLC) retention times) confirmed that PHF derived from parathyroid cell cultures was identical (303) to that isolated from the plasma of SHR (311) and low-renin hypertensive humans (312).

PHF was ultimately also shown to be elevated in other low-renin rat models of hypertension (313, 314), hypertensive primary hyperparathyroid patients (282), and low-renin hypertensive patients (312). Subsequently, PHF has been detected in plasma of normotensive humans (315), although the physiological role of PHF is unknown. Since PHF was shown to be released from the PTG, a tissue which is negatively regulated by plasma Ca^{2+}, the efficacy of dietary calcium in low-renin hypertension can be explained by the properties of PHF.

1.4.3 Mechanism of action

Concurrent with the blood pressure studies of SHR plasma extracts, were vascular smooth muscle Ca^{2+} uptake studies. Partially purified plasma from SHR, but not WKY rats, enhanced Ca^{2+} uptake into isolated rat tail arteries derived from normotensive animals (302). Subsequent studies demonstrated that although PHF did not have direct effects on L-type Ca^{2+} channels of VSMC, PHF enhanced both the influx and the rise in intracellular Ca^{2+} induced by NE and KCl (316). PHF was also shown to potentiate the pressor effects of classical vasoconstrictors *in vivo* (317). However, in red blood cells, PHF was shown to directly stimulate an increase in cytosolic Ca^{2+} (318). These findings are supported by the observation that the L-type Ca^{2+} channel blocker, nifedipine, inhibited the pressor effects of PHF in the blood pressure bioassay (316). The time courses for all of these effects are consistent with that of PHF in the blood pressure bioassay with peak activity occurring between 45-60 minutes. Based on this proposed mechanism of action, it is not surprising that plasma PHF levels are able to predict the efficacy of Ca^{2+} channel blockers in treating essential hypertension (319). The actions of PHF are not confined to VSMC as PHF has been implicated in other conditions which exhibit pathological elevations in intracellular Ca^{2+} in the relevant tissues. For example, elevated PHF has been shown in a significant proportion of patients with non-insulin-dependent diabetes mellitus (320).

Additional mechanisms of action for PHF have been investigated including stimulation of phosphodiesterase (PDE) activity. PDE catalyzes the cleavage of the cyclical form of cAMP to the linear, inactive form. A decrease in cAMP is associated with increased contractility in vascular smooth muscle. PHF has been shown to stimulate PDE activity, thus decreasing cAMP and contributing to vasoconstriction (316).

A third mechanism of action of PHF involves regulation of K^+ channels. PHF has been shown to inhibit the delayed rectifier type potassium current with the same time course as for PHF-induced effects on Ca^{2+} uptake and blood pressure (321). This inhibition of the delayed rectifier K^+ channel was associated with a decrease in the resting membrane potential suggesting that PHF may contribute to enhanced vascular contractility by bringing the membrane potential closer to that required for activation of L-type Ca^{2+} channels.

1.4.4 Involvement of PHF in low-renin hypertension

Studies utilizing parathyroidectomy and PTG cross-transplantation would seem to indicate the importance of PHF in the progression or maintenance of low-renin hypertension. These studies showed not only the linkage between the PTG and hypertension, but that appearance of plasma PHF could be correlated with hypertension. None-the-less, these data do not unequivocally demonstrate the fundamental role of PHF in the pathology of hypertension. Recently the important role of PHF in hypertension has been demonstrated using antisera to PHF. Injection of this antisera reduced mean arterial blood pressure to within a normal range (approximately 110 mmHg) in SHR and pre-incubation of the antisera with partially purified PHF abolished the pressor activity of PHF in the blood pressure bioassay (322). Subsequent studies performed using the purified monoclonal anti-PHF antibody (described below) have confirmed these results. This is the strongest evidence for the role of PHF in hypertension to date. Until recently, PHF was only detected in biological samples derived from SHR animals suggesting that PHF may represent some aberrant by-product of PTG overactivity in low-renin hypertension. However, PHF is now detectable in parathyroid cell and organ cultures derived from normotensive WKY rats and in the plasma of normotensive humans (315). The physiological role of PHF is purely speculative, but may represent the first description of a systemic regulator of intracellular calcium. PHF physiology will be explored in greater detail in the Discussion section of this thesis.

1.4.5 PHF Structure

Recently a monoclonal antibody has been raised against PHF which has been used for a variety of applications. An ELISA for the detection of PHF has been developed which has simplified the quantitation of PHF levels in biological samples (315, 323), which were previously measured by bioassay. The monoclonal antibody has also been used for immunocytochemistry and immunohistochemistry (reported here), Western blotting, and affinity purification of PHF. The structure of PHF prior to production of affinity-purified PHF was unknown but believed to be a peptide-lipid complex of approximately 3-4 kDa (306). However, subsequent studies of chromatography purified PHF have indicated a structure of approximately 4 kDa. This material is currently being purified in mass production to obtain adequate concentrations to allow peptide sequencing.

1.4.6 Regulation of PHF secretion

The regulation of PHF synthesis and secretion is poorly understood. Since the low-renin subset of hypertension coincides with the salt-sensitive subset, and normotensive rats could be made hypertensive by desoxycorticosterone acetate-salt (DOCA-NaCl) treatment, it is logical to examine the effect of salt intake on plasma PHF. Several dietary studies have revealed that a high salt intake may increase plasma PHF, or PHF-like bioactivity in human essential hypertension (241, 312). Dietary salt loading also induced expression of PHF in the plasma of Dahl-SS hypertensive rats (314), and stimulated PHF-like bioactivity in SHR animals (309).

Since PHF was shown to originate in the PTG, it was also hypothesized that calcium would negatively regulate PHF as for PTH. Inhibition of PHF synthesis or secretion by calcium would account for the efficacy of dietary calcium supplementation in treatment of low-renin hypertension. Dietary studies in both SHR and DOCA-NaCl hypertensive rats showed that increased calcium intake was associated with lower plasma PHF or PHF-like pressor activity (324, 325). However dietary studies with calcium, although useful clinically, introduce multiple variables (including decreased plasma 1,25-$(OH)_2D_3$) which may confound the picture and thus do not provide evidence of a direct effect of EC Ca^{2+} on PHF synthesis or secretion. As a result, studies were undertaken utilizing parathyroid organ cultures derived from SHR and WKY rats with PHF detected by the blood pressure bioassay. The results yielded two important pieces of information: first, that reduction of Ca^{2+} in culture media was associated with detectable PHF, and second, that WKY parathyroid organ cultures did not produce detectable PHF (303). The recent development of the PHF enzyme-linked immunosorbant assay (ELISA) has enabled more precise studies of the direct effects of EC Ca^{2+} on PHF release from cultured intact PTG or dispersed parathyroid cells.

1.5 Summary

The parathyroid glands represent the control center for calcium and phosphate metabolism. They sense minute changes in EC Ca^{2+} through the CaR and respond with changes in PTH synthesis and secretion. If necessary, the PTG can also be stimulated to proliferate as part of an appropriate response to hypocalcemia and vitamin D3 deficiency (as in secondary hyperparathyroidism) or an inappropriate response in the face of hypercalcemia (as in primary hyperparathyroidism). There is a clear association between hyperparathyroidism and hypertension. A substantial proportion of primary hyperparathyroid patients are hypertensive and similarly, the low-renin subset of hypertensives is characterized by abnormalities in calcium homeostasis indicative of secondary hyperparathyroidism. Specifically, low-renin hypertension displays hypocalcemia and elevations in both PTH and 1,25-$(OH)_2D_3$. Studies of this apparent relationship between hyperparathyroidism and hypertension have indicated a role of the PTG in hypertension. Parathyroid abnormalities such as hyperplasia and the detection of a novel cell type have been observed in the SHR (a model of low-renin hypertension), however the factors which contribute to these anomalies are unknown. Parallel to these parathyroid studies, a paradox emerged in which both dietary calcium and Ca^{2+} channel blockers were effective in ameliorating low-renin hypertension. This paradox could be resolved by the introduction of the "parathyroid hypertensive factor"; a vasopressor substance which is secreted from the PTG and appears to act through potentiation of L-type Ca^{2+} channel currents. PHF was found to be elevated in low-renin experimental and human hypertension. Dietary studies and parathyroid organ culture studies have suggested that EC Ca^{2+} may directly inhibit secretion of PHF, but the precise regulation of PHF synthesis and secretion is largely unknown. Although PHF structure remains elusive, the development of an ELISA for PHF detection has allowed for the pursuit of the potential regulators of PHF secretion. Identification of key regulators of PHF release and characterization of the signal transduction pathways they utilize will have important therapeutic potential for the development of antagonists of PHF secretion.

1.6 Hypothesis

Based on the literature reviewed above, my hypothesis for this thesis is as follows. Parathyroid cells derived from SHR share several features with the parathyroid cells of hyperparathyroidism, however do not display features consistent with a transformation to a cancerous phenotype. In addition, all parathyroid tissue secretes PHF, however SHR PTG secrete more PHF than WKY PTG. This release is inhibited by EC Ca^{2+} and stimulated by 1,25-$(OH)_2D_3$ based on the observed plasma ionic and hormonal profiles of low-renin hypertension and the fact that both EC Ca^{2+} and 1,25-$(OH)_2D_3$ are key parathyroid regulators. Furthermore, to account for the elevated plasma PHF of the SHR, parathyroid cells of SHR secrete more PHF and proliferate to a greater extent than WKY partially due to increased sensitivity to stimulation by low EC Ca^{2+} and 1,25-$(OH)_2D_3$. This differential sensitivity is related to alterations in expression of the receptors which mediate the effects of EC Ca^{2+} and 1,25-$(OH)_2D_3$; namely, the CaR, $1{,}25VDR_{mem}$, and the $1{,}25VDR_{nuc}$.

1.7 Aims of the thesis

The aims of this thesis are diagramed in Figure 1-3 and are as follows:

1. To characterize the parathyroid cell biology of the current model of primary parathyroid cell culture derived from SHR and WKY rats. The proliferative and metabolic properties will be compared and contrasted as will the response to serum-deprivation induced cell death. In addition, the regulation of

parathyroid cell viability by EC Ca^{2+} and 1,25-$(OH)_2D_3$ will be examined and compared between the two strains.

2. To confirm and extend the information suggesting an inhibitory effect of EC Ca^{2+} on PHF secretion. Parathyroid cell and organ cultures will be exposed to varying concentrations of EC Ca^{2+} under various conditions to better understand the complex regulation of PHF release by Ca^{2+}. Furthermore, the role of the CaR in mediating these potential effects will be examined and any strain differences in sensitivity to EC Ca^{2+}, which may be related to CaR expression, will be noted.
3. To examine the effect of vitamin D3 on PHF secretion. Parathyroid cell and organ cultures will be exposed to 1,25-$(OH)_2D_3$ (and other vitamin D3 analogs as relevant), under various conditions to determine the role of 1,25-$(OH)_2D_3$ in regulation of PHF release. The mechanism of potential effects will be investigated including the roles of the two VDR, 1,25VDR_{mem} and 1,25VDR_{nuc} in mediating these effects.

1.8 Claims of Originality

The novel findings of this thesis are as follows:

1. SHR PTC display abnormalities suggestive of a hyperplastic cell condition. These abnormalities include increased proliferation rate and growth to a greater plateau cell density and possibly increased metabolic rate and resistance to spontaneous apoptosis under normal culture conditions.

2. Acute exposure to EC Ca^{2+} under high density culture conditions results in an enhanced number of viable cells for both SHR and WKY PTC. Similarly, acute and to a greater extent, sub-acute exposure to 1,25-$(OH)_2D_3$ results in an increase in the number of viable cells for both SHR and WKY PTC.
3. SHR and WKY PTC are not differentially regulated by EC Ca^{2+} with respect to effects on the number of viable cells. Differential effects are observed however with 48 hours of 1,25-$(OH)_2D_3$ treatment. These differences are likely secondary to the increased proliferation rate of SHR PTC.
4. Normal parathyroid tissue (represented by WKY PTC and PTG organ cultures) secretes PHF.
5. SHR parathyroid tissue produces more PHF under basal conditions than WKY PTC suggesting the existence of some intrinsic abnormality in PHF release in the SHR PTG.
6. Acute exposure to low EC Ca^{2+} stimulates PHF release from both SHR and WKY parathyroid tissue. For cultured PTC, significant stimulation of PHF release by low EC Ca^{2+} occurs at approximately the same cell density in culture for SHR and WKY PTC.
7. Both WKY and to a greater extent, SHR PTC express CaR protein. Decreased expression of this protein is associated with low EC Ca^{2+} and a rapidly proliferating cell population.
8. The CaR appears to mediate the effects of EC Ca^{2+} on PHF release in both SHR and WKY PTC.
9. Acute and sub-acute exposure to 1,25-$(OH)_2D_3$ stimulates PHF release in a

dose-dependent and $1,25VDR_{nuc}$–independent manner for both SHR and WKY PTC.

10. Acute and sub-acute exposure to $24,25\text{-}(OH)_2D_3$ inhibits PHF release in a dose-dependent manner for both SHR and WKY PTC.
11. Rapid stimulation of PHF release by $1,25\text{-}(OH)_2D_3$ occurs with increased sensitivity in SHR PTC, whereas rapid inhibition of PHF release by $24,25\text{-}(OH)_2D_3$ occurs with decreased sensitivity in SHR PTC.
12. Both WKY, and to a greater extent, SHR PTC express $1,25VDR_{mem}$ protein. Similar amounts of the $1,25VDR_{nuc}$ protein are observed in SHR and WKY PTC.

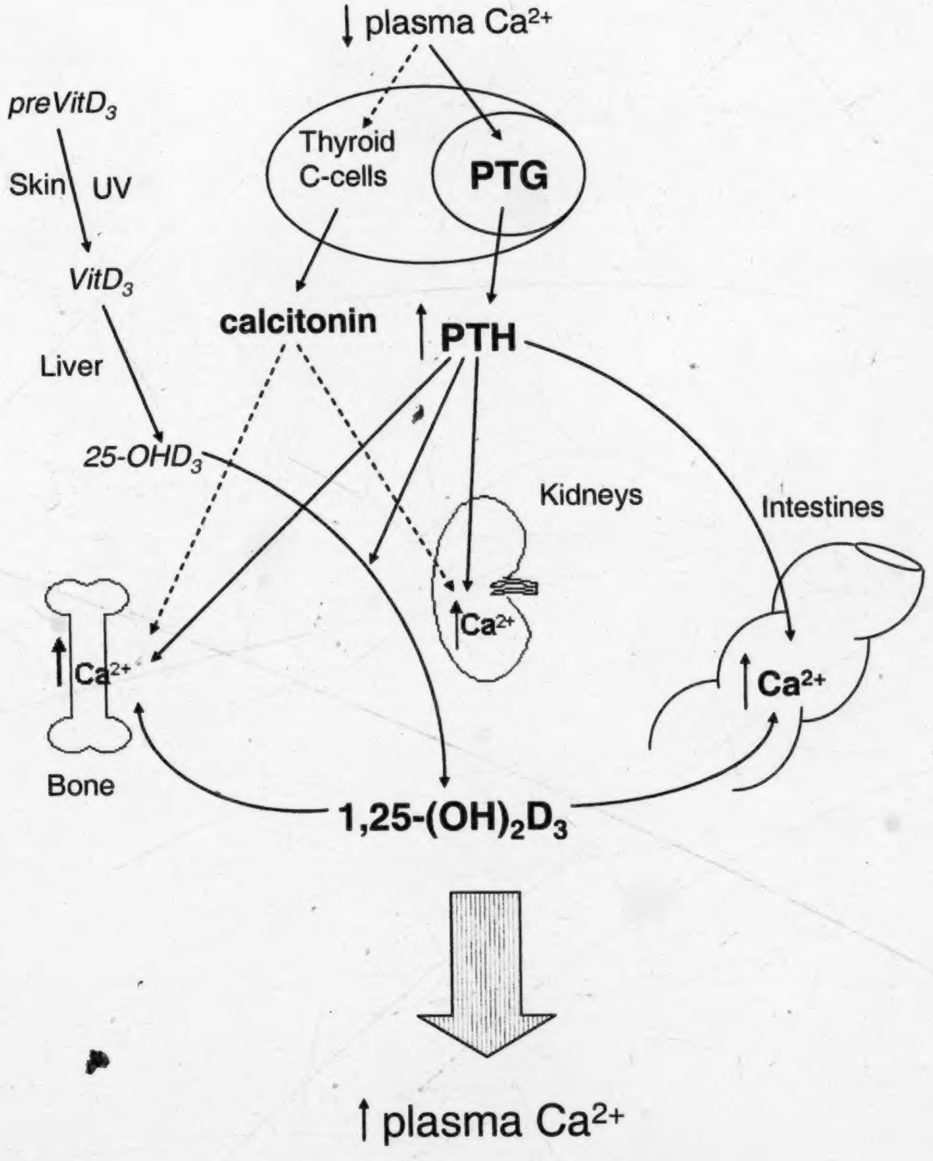

↓ plasma Ca^{2+}
$preVitD_3$
Skin
UV
Thyroid C-cells
PTG
$VitD_3$
calcitonin
↑ PTH
Liver
$25\text{-}OHD_3$
Kidneys
Intestines
↑ Ca^{2+}
↑ Ca^{2+}
↑ Ca^{2+}
Bone
$1,25\text{-}(OH)_2D_3$
↑ plasma Ca^{2+}

Figure 1-1—Overview of calcium homeostasis. Solid lines indicate activation or stimulatory effects and dashed lines indicate inhibitory effects.

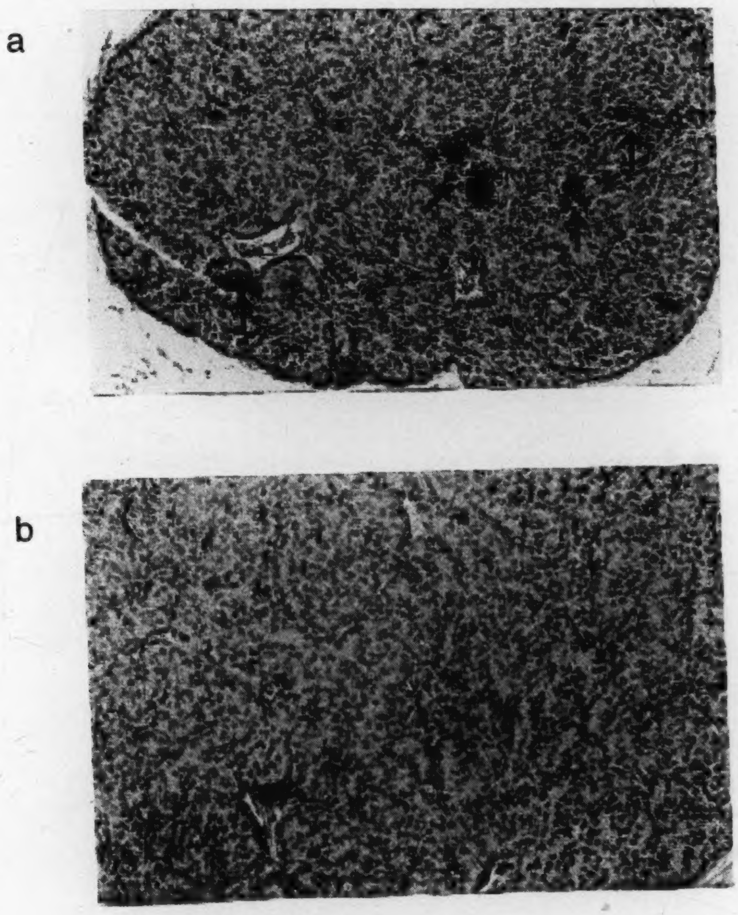
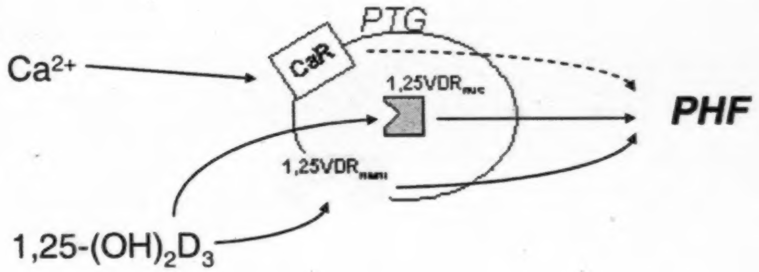


Figure 1-2—Novel cell localization in the SHR parathyroid. Parathyroid sections from SHR (a) and WKY (b) rats were stained with hematoxylin and eosin. Arrows indicate clusters of darkly stained "novel" cells, which are visible in SHR, but not WKY, parathyroid sections (Unpublished results of histochemistry performed by Toyoji Kaneko).

WKY


Ca2+
CaR
PTG
1,25VDRnuc
1,25VDRmem
PHF
1,25-(OH)2D3

Developing SHR

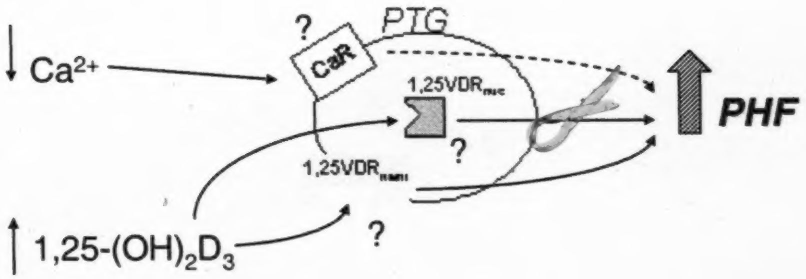

?
Ca2+
CaR
PTG
1,25VDRnuc
?
1,25VDRmem
PHF
1,25-(OH)2D3
?

Figure 1-3—Regulation of PHF secretion: hypothesis. Solid lines indicate activation or stimulatory effects and dashed lines indicate inhibitory effects. Question marks indicate unknown alterations in receptor protein expression levels in SHR parathyroid cells

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Chapter 2

Abnormal growth properties of parathyroid cells derived from spontaneously hypertensive rats and regulation by extracellular calcium and 1,25-dihydroxyvitamin D3[1]

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2 ABNORMAL FEATURES OF SHR PARATHYROID CELLS

2.1 Introduction

The parathyroid glands, like many endocrine tissues, exhibit a low cell turnover rate and long cell lifespan. Several key physiological regulators function to either maintain this net zero growth or to induce proliferation under conditions which require enhanced PTH secretion, namely hypocalcemia and vitamin D3 deficiency. Low EC Ca^{2+} can stimulate an increase in PTC proliferation (1-3) via the CaR (4). The biologically active vitamin D3 metabolite, 1,25-$(OH)_2D_3$, has not always been shown to exert effects on parathyroid proliferation *in vivo* (3), however inhibitory effects have clearly been demonstrated *in vitro* (5). A third emerging regulator which is receiving increasing attention for its importance in chronic renal failure is phosphate. Dietary studies have shown that phosphate stimulates parathyroid cell proliferation in both normal rats (6) and experimental models of hyperparathyroidism *in vivo* (3) and direct effects have been confirmed *in vitro* in parathyroid cell cultures (7).

Abnormal parathyroid proliferation is one of the hallmarks of both primary and secondary hyperparathyroidism. The pathogenesis of hyperplasia in secondary hyperparathyroidism due to renal failure has been reviewed elsewhere (8) but appears to involve a variety of factors; several of which also apply to primary hyperparathyroidism. Not surprisingly, abnormalities in cell cycle regulation in some parathyroid adenomas have been reported (for review, see ref (9)) and mutations of oncogenes have also been suggested for the hyperplasia of secondary hyperparathyroidism (10). In addition to these compounding genetic factors, hypocalcemia and vitamin D3 deficiency due to renal disease may further exacerbate the parathyroid hyperplasia. The CaR appears to play a key role in the mechanism of hypocalcemia-induced secondary hyperparathyroidism due to chronic renal insufficiency (4). Downregulation of the CaR would then present one mechanism by which parathyroid hyperplasia could develop and in fact, several studies have indicated reduced parathyroid expression of the CaR in both primary (11, 12) and secondary hyperparathyroidism (13). In a similar manner, both vitamin D3 deficiency and the reported downregulation of the classical $1,25VDR_{nuc}$ in parathyroid tissue of chronic renal failure patients (14) and experimental animals (15) may contribute to the pathogenesis of hyperparathyroidism.

Some of the features of secondary hyperparathyroidism are manifested in low-renin hypertension. This subset of hypertension, representing approximately 30-40% of all essential hypertension, exhibits hypocalcemia accompanied by appropriate shifting of the calcium-regulating hormones, i.e. increased plasma PTH, elevated $1,25\text{-}(OH)_2D_3$, and suppressed calcitonin (16). Since low-renin hypertension is accompanied by elevated PTH, it is reasonable to predict that another feature of hyperparathyroidism, parathyroid hyperplasia, may also be present. Very little is known about parathyroid cell turnover in low-renin hypertension. The only study to date published on the subject revealed that parathyroids derived from SHR (a hypertensive model with reduced plasma renin activity (17)), had increased parathyroid wet weight, due to hyperplasia rather than hypertrophy, compared with parathyroids taken from the normotensive genetic control WKY rats (18). In addition, the PTG of SHR but not WKY rats express a distinct cell type (denoted as "novel cells"), the percentage of which was found to be correlated with the magnitude of hypertension (19). These cells were also correlated with plasma levels of PHF, a substance found to be elevated and believed to play a causal role in low-renin hypertension (for review, see (20)).

We have previously shown that secretion of PHF from cultured SHR and WKY parathyroid cells is stimulated by 1,25-$(OH)_2D_3$ (21) (see section 5.3.1) and low EC Ca^{2+} (22) (see section 4.3.1) as measured by ELISA for PHF. Here we describe their roles in regulation of SHR and WKY parathyroid cell proliferation in an *in vitro* culture system. We also characterized the cell growth properties and responses to induced cell death in this system and show that SHR parathyroid cells share many features with cultured parathyroid cell lines developed from rat experimental models of secondary hyperparathyroidism. The results support the hypothesis that SHR parathyroid cells and abnormal regulation thereof may be contributing to the elevated plasma PHF observed in this model through an overall increase in parathyroid mass.

2.2 Materials & Methods

2.2.1 Materials

Vitamin D3 analog 1,25-dihydroxyvitamin D3 was obtained from Roussell UCLAS and Sigma Aldrich Chemical Company (St. Louis, MO, USA) and 1,24$(OH)_2$-22-ene-24-cyclopropyl-D_3 (BT) was a generous gift from Leo Pharmaceuticals (Copenhagen, Denmark). All cell culture media were obtained from Gibco/BRL (Burlington, ON). WST-1 cell proliferation reagent, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI), and annexin-V-FLUOS were obtained from Roche Biochemicals (Mannheim, Germany). Vectashield was obtained from Vector

Laboratories (Burlingame, CA, USA). All other chemicals were purchased from either Sigma/Aldrich (St. Louis, MO, USA) or Fisher chemicals (Fairlawn, NJ, USA).

2.2.2 Preparation of Parathyroid Cell Cultures and Determination of Parathyroid Cell Viability

Parathyroid cell suspensions in DMEM supplemented with 5% FBS were obtained as previously described (23). Briefly, thyroparathyroid complexes were removed from male 12-14 week old SHR and WKY rats, washed in ice-cold Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/neomycin (PSN), and parathyroid tissue dissected and minced and placed in a minimal amount of medium to allow attachment to the culture flasks. Following attachment of the explants (approximately 48 hours later), additional culture medium was added and cells were maintained in 15% FBS in DMEM until confluent. A continuous cell culture was established by harvesting following trypsin-induced detachment and plating in 10% FBS in DMEM.

Parathyroid cell number was determined by using either the trypan blue exclusion method or the WST-1 cell proliferation assay. For cell number determination on 96-well culture plates, 10 μl WST-1 reagent was added to each well (containing 100 μl of 1.5 mM Ca^{2+} phenol red-free Ham's F12 medium + 5% FBS) and the plates incubated 2 hours at 37 °C, 5% CO_2. Optical density at 450 nm (with reference wavelength of 650 nm) was then measured by a Labsystem Multiskan MCC microplate reader. Absorbance was compared to a cell standard curve prepared just prior to addition of WST-1 (allowing a 4 hour attachment period), with cell number determined by the trypan blue exclusion method. For all other experiments, parathyroid cell suspensions were obtained by trypsin treatment and cell viability determined using the trypan blue exclusion method.

2.2.3 Morphological Characterization of Cell Death

Serum is required for normal cell proliferation and for the prevention of apoptosis. Removal of essential growth factors and serum components such as iron results initially in cell cycle arrest, followed by induction of apoptosis. To compare the response to serum-induced apoptosis in SHR and WKY PTC, parathyroid cell suspensions in DMEM supplemented with 5% FBS were obtained as described above. Cells were seeded at an intermediate cell density directly onto coverslips and allowed to attach for 48 hours. Medium was then changed to Ham's F12 medium adjusted to 1.5 mM Ca^{2+} supplemented with either 5% FBS or 0.2% FBS to induce apoptosis and incubated for 96 hours at 37 °C, 5% CO_2. To label all cell nuclei, coverslips were washed once with 1 μg/ml DAPI in methanol and then incubated with 1 μg/ml DAPI in methanol for 15 minutes at 37 C in darkness. To label dying cells, coverslips were exposed to 1:50 diluted annexin-V-FLUOS labeling reagent and 1 μg/ml propidium iodide in an incubation buffer containing 10 mM Hepes/NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4 for 10 minutes at room temperature. Coverslips were then rinsed in PBS, mounted on slides with Vectashield and photographed using a SPOT-2 digital camera and an Olympus BX40 fluorescence microscope.

2.2.4 Regulation of Parathyroid Cell Viability by Vitamin D3 Analogs and EC Ca^{2+}

For investigation of the effects of EC Ca^{2+} and different vitamin D3 analogs on parathyroid cell viability at various cell densities, cell suspensions in DMEM supplemented with 10% FBS were obtained as previously described (23), seeded in 96-well plates for approximately 48 hours to achieve a density of 0.3, 2, and 4 x 10^4 cells/well representing low (LDCC), intermediate (IDCC), and high (HDCC) density culture conditions. The effect of EC Ca^{2+} was examined by changing media to control medium (Ham's F12 medium supplemented with 5% FBS) adjusted to 0.5 mM (low), 1.2 mM (normal), or 1.8 mM (high) EC Ca^{2+} in the presence or absence of 10 μM cytosine-arabinoside and incubating plates for a further 4 or 48 hours at 37 °C, 5% CO_2. The effect of vitamin D3 analogs was examined as for EC Ca^{2+} experiments. Analogs were prepared in ethanol and diluted in 1.5 mM Ca^{2+} control medium so that the final concentration of ethanol was less than 0.1% (v/v).

2.2.5 Statistical Analysis

Results are expressed as the mean ± SEM and statistical evaluation for differences between treatment groups was performed using t-tests, one-way ANOVA, or two-way ANOVA where appropriate.

2.3 Results

2.3.1 Basal Metabolic Rate in Sub-cultured SHR and WKY Parathyroid Cells

When experiments utilizing SHR and WKY PTC were initially performed, differences were observed in the absorbance in the WST-1 proliferation assay for the same number of SHR and WKY PTC. Comparison of the cell standard curves for SHR and WKY PTC are shown in Figure 2-1. Since the cellular mitochondrial enzymes are responsible for the metabolism of WST-1 to the colored formazan product, increased absorbance generated by the SHR PTC standard curve versus the WKY PTC standard curve may indicate an increased basal metabolic rate by SHR PTC.

2.3.2 Comparison of SHR and WKY Parathyroid Cell Proliferation Rate

SHR and WKY parathyroid cell growth over time is shown in Figure 2-2. Both SHR and WKY PTC were seeded at 0.2 x 10^4 cells/well. After 1 day to allow attachment, the number of viable PTC was equal to or exceeded 0.2 x 10^4 cells/well for WKY and SHR PTC respectively indicating that PTC derived from both strains initially attached to the plates and exhibited high viability. SHR parathyroid cells were shown to proliferate at a much faster rate during both the early linear phase of growth and the later exponential phase and reached a higher plateau cell number. SHR PTC doubling time was significantly shorter than that of WKY PTC (30 vs 54 hours) whereas plateau cell density was increased (approximately 5 x 10^4 cells/well vs 2 x 10^4 cells/well). These values for SHR PTC were somewhat comparable with those reported for PT-r cells, a model of vitamin D3 deficiency-induced secondary hyperparathyroidism (24). The doubling time for 4 different clonal PT-r cell lines ranged from 14-20 hours and, when adjusted for a 96-well surface area, the calculated plateau cell density was approximately 7 x 10^4 cells/well.

2.3.3 Effect of Serum Deprivation on SHR and WKY Parathyroid Cell

Death

Serum starvation of PTC derived from SHR and WKY rats resulted in growth restriction and/or cell death to approximately the same final cell number for SHR and WKY PTC (Figure 2-3). However, the extent of this reduction was greater for SHR (31.7% vs 23.0% decrease) than WKY PTC due to a greater final cell number in the SHR PTC control 5% FBS group.

Apoptosis is associated with cellular changes such as nuclear condensation and fragmentation. The effect of serum deprivation on PTC nuclear morphology was studied utilizing the nuclear stain, DAPI. Figure 2-4 shows DAPI-stained nuclei from SHR (a, b) and WKY (c, d) PTC grown in normal (a, c) and serum-starved (b, d) conditions. Fragmented and condensed nuclei were seen in similar proportions for both SHR and WKY PTC under both serum-starved and normal serum conditions. As observed with WST-1 studies, SHR PTC proliferated to a greater extent over the 96 hour period than the WKY PTC.

Figure 2-5 shows cell death under both normal and serum-deprived conditions for SHR (a-d) and WKY (e-h). Apoptotic cell death was detected through annexin-V-Fluos labeling of phosphatidylserine residues. These residues are translocated from the inner to the outer leaflet of the lipid bilayer of the cell plasma membrane during early apoptotic cell death, however since the membrane integrity is disrupted during necrotic cell death, annexin-V-Fluos can permeate and stain necrotic cells. To distinguish apoptotic and necrotic cell death, the nuclear stain propidium iodide, which can only permeate necrotic cell membranes, was used. The staining patterns indicated that low levels of apoptosis and necrosis were observed in SHR PTC under normal serum conditions (Fig. 2-5 a, b), however apoptosis and necrosis were seen to a greater extent in WKY PTC under normal serum conditions (Fig. 2-5 e, f). Under serum-starved conditions, both SHR and WKY PTC exhibited apoptosis and necrosis to a similar extent (Fig. 2-5 c, d, g, h). These results are in agreement with those obtained using WST-1 detection of cell viability and suggest that the reduced cell number for WKY PTC under normal serum conditions may be related to increased cell death versus SHR PTC.

2.3.4 Effect of EC Ca^{2+} on Parathyroid Cell Viability

The effect of EC Ca^{2+} on PTC viability was examined at various time points and cell culture densities. Cells were seeded at various densities and allowed to completely attach to the culture plates for 48 hours before initiation of an experiment. Following the attachment phase, exposure to various concentrations of EC Ca^{2+} for 4 hours resulted in a significantly greater number of viable parathyroid cells in the lowest (0.5 mM) EC Ca^{2+} group for both the IDCC and HDCC groups for SHR PTC, but only the HDCC group for WKY PTC (Figure 2-6a). The maximum percentage change in cell number was nearly 20% which was deemed appropriate for the 4 hour incubation period based on doubling time calculations. It was expected that a greater change in cell number would occur following exposure to different concentrations of EC Ca^{2+} for 48 hours. However, in both SHR and WKY PTC, no significant effects were observed (Figure 2-6b).

In order to determine whether the effect of EC Ca^{2+} was due to stimulation of proliferation by the low EC Ca^{2+} group or induction/enhancement of cell death by the higher EC Ca^{2+} groups, higher density SHR and WKY PTC cultures were exposed to various concentrations of EC Ca^{2+} in the presence or absence of the mitotic inhibitor, cytosine-arabinoside. Figure 2-7 shows that a small, but significant inhibitory effect of EC Ca^{2+} on PTC number was maintained in the presence of cytosine-arabinoside for both SHR and WKY PTC. Incubation of cell cultures with 10 μM cytosine-arabinoside for 48 hours arrested proliferation of cells, with minimal cell death (data not shown). Since all proliferation was prevented by cytosine-arabinoside, the effect of EC Ca^{2+} on PTC number was due to induction or enhancement of parathyroid cell death.

2.3.5 Effect of 1,25-$(OH)_2D_3$ and Analog BT on Parathyroid Cell Viability

The effect of 1,25-$(OH)_2D_3$ on PTC viability was examined as for EC Ca^{2+}. Exposure to 100 nM 1,25-$(OH)_2D_3$ for 4 hours resulted in a small, but significant stimulation of PTC viability for both the IDCC and HDCC groups for SHR PTC, but only the HDCC group for WKY PTC (Figure 2-8a). After 48 hours however, a marked stimulation of cell number was observed in the LDCC groups for both SHR and WKY PTC, in addition to small but significant stimulatory effects in the IDCC and HDCC WKY PTC groups (Figure 2-8b).

To examine whether 1,25-$(OH)_2D_3$ was able to alter PTC number in the absence of proliferation and also to address the potential role of the $1,25VDR_{nuc}$ in mediating the effects after 48 hours, we exposed cells to the mitotic inhibitor, cytosine-arabinoside, as for EC Ca^{2+} studies, in the presence of absence of 1,25-$(OH)_2D_3$ or the $1,25VDR_{nuc}$–specific vitamin D3 agonist, BT. Figure 2-9a shows that both 1,25-$(OH)_2D_3$ and analog BT significantly stimulated SHR PTC viability which was abolished in the presence of cytosine-arabinoside. No effects of either 1,25-$(OH)_2D_3$ or analog BT were observed for WKY PTC (Figure 2-9b). Since proliferation did not occur over the 48 hour time period for WKY PTC, the effects of 1,25-$(OH)_2D_3$ are likely cell growth-dependent for these cells as well.

2.4 Discussion

In this study, parathyroid cells derived from the hypertensive model SHR, which exhibits some features of secondary hyperparathyroidism, were compared with cells derived from the normotensive control strain, WKY rats, and the effects of EC Ca^{2+} and vitamin D3 analogs in regulation of cell proliferation were examined. Cultured SHR PTC were shown to proliferate much faster and to a greater saturation density versus WKY parathyroid cells. Low EC Ca^{2+} and 1,25-$(OH)_2D_3$ were both shown to enhance parathyroid cell viability by different mechanisms. The results from this study suggest that the parathyroid tissue of the SHR may be hyperplastic which may result in enhanced maximal PHF secretion and lead to the elevated plasma PHF observed in this model. PHF secretion studies under both basal conditions and in response to EC Ca^{2+} and vitamin D3 metabolites are reported in subsequent chapters of this thesis.

SHR parathyroid cells grew at a much faster rate than WKY parathyroid cells supporting an earlier study which suggested that SHR PTG were hyperplastic (18). Increased proliferation rates have been observed in other SHR tissues such as vascular smooth muscle (25). The PTC growth curves generated in this study were compared with those of subclones derived from the rat parathyroid cell line, PT-r (26). This line is derived from a rat model of secondary hyperparathyroidism induced by vitamin D3 deficiency. The range of doubling times for PT-r subclones was 14-20 hours. This was more comparable with the SHR PTC doubling time of 30 hours than the WKY PTC doubling time of 54 hours. The saturation or plateau density reported for the hyperplastic PT-r subclones was also much closer to the saturation density observed for SHR PTC than for WKY PTC. Furthermore, cultured parathyroid cells from the normotensive WKY rats became senescent after several weeks in culture indicated by a cessation in growth and secretion of PHF, however SHR parathyroid cells continued to secrete PHF and grow to a much greater extent. We also observed that SHR parathyroid cells metabolized the tetrazolium salt, WST-1, to a greater extent than the same number of WKY cells. Since WST-1 is broken down by the mitochondrial enzymes involved in cellular metabolism, this indicates that SHR PTC may have a higher basal metabolic rate than WKY PTC. These features are all consistent with the hypothesis that SHR parathyroid tissue is hyperplastic.

The finding that SHR PTC grew to a greater plateau density prompted the question of whether these cells were abnormally resistant to contact inhibition and apoptosis. Serum deprivation experiments utilizing WST-1 indicated that SHR PTC underwent a greater decrease in cell number than WKY PTC seeded at the same starting density. This likely was due to a combination of increased proliferation by SHR PTC, and greater apoptosis for WKY PTC under normal serum conditions as suggested by increased annexin-labeling. Cell morphology after serum deprivation was examined and compared between the two cell lines by utilizing the nuclear specific dye, DAPI. The results confirmed those from the WST-1 studies. To determine whether this serum-deprivation induced cell death was apoptotic or necrotic in nature, we utilized annexin and propidium iodide staining of necrotic cells. These studies revealed that the majority of cell death was apoptotic in nature and that SHR and WKY parathyroid cells responded to serum starvation with similar levels of apoptosis. The relevance of these *in vitro* observation to an *in vivo* setting is unclear since normal parathyroid tissue undergoes a relatively slow rate of cell turnover (27). Several *in vivo* studies which involved nutritional induction of secondary hyperparathyroidism in rats found no detectable apoptosis in either hyperplastic or normal parathyroid tissue (3, 6, 28). This may be due to the nature of the *in vivo* dietary studies which examined endpoint PTG tissue for evidence of apoptosis—a process which likely occurs at a low level and over relatively short time periods. However, subsequent studies have shown that apoptosis occurs at a comparable level to proliferation in normal parathyroid tissue, and at a higher rate in hyperplastic parathyroid tissue (29). A higher rate of apoptosis in the hyperplastic parathyroid of secondary hyperparathyroidism (29) is more likely due to increased cell turnover and is secondary to the increase in proliferation. However, we observed no difference in apoptosis between SHR and WKY parathyroid cells. In fact, SHR parathyroid cells were shown to be more resistant to apoptosis under normal conditions than WKY cells. This discrepancy may be due to inherent differences in the parathyroid of low-renin hypertension versus the parathyroid of secondary hyperparathyroidism, or alternatively, may be an *in vitro* observation which is not reflected *in vivo*.

Based on previous studies, low EC Ca^{2+} was hypothesized to stimulate parathyroid cell proliferation. Our studies showed that low EC Ca^{2+} indeed enhanced cell viability under HDCC, but the effect of calcium was not prevented by chemical blockade of proliferation by the anti-mitotic agent, cytosine-arabinoside. This indicates that high EC Ca^{2+} either induced parathyroid cell death, or enhanced apoptosis induced by the high-density culture system. High EC Ca^{2+} was also shown to inhibit parathyroid cell viability at acute time points, but not by 48 hours; an observation which was also seen in studies of regulation of PHF secretion (30). The cause of this loss of sensitivity to EC Ca^{2+} is unknown but may be related to cellular changes which are induced by short term exposure to high EC Ca^{2+}, or may be due to biphasic effects of EC Ca^{2+} in this culture system. Although it is widely accepted that hypocalcemia stimulates proliferation *in vivo*, the *in vitro* effects of EC Ca^{2+} on parathyroid cell proliferation are not as clear. In addition to the predicted inhibitory effects (24), there have been reports of EC Ca^{2+} stimulating proliferation (7), biphasic effects (1, 7), and no effects (31). It is possible that EC Ca^{2+} influences multiple pathways in the control of proliferation *in vitro* and that there is a balance of both stimulatory and inhibitory effects. In fact, Roussanne et al have suggested that EC Ca^{2+} may both inhibit and stimulate parathyroid cell proliferation depending on whether CaR expression is high or low respectively (7) (CaR protein levels in the SHR and WKY PTC culture system are reported in Chapter 4). These findings may explain the small magnitude of change in cell number at 4 hours, and the lack of difference in cell number at 48 hours.

Although bovine parathyroid cells lose the ability to be regulated by EC Ca^{2+} (32) related to downregulation of the CaR, several other long-term parathyroid cell culture systems in addition to our own model (30), have successfully retained regulation by EC calcium, and when examined, expression of the CaR (1, 24, 33). We've previously shown that expression of the CaR protein is increased in cultured SHR PTC and that CaR protein is reduced at lower cell densities which normally exhibit parathyroid proliferation versus high-density, quiescent populations (22). Brown et al concluded that downregulation of the CaR is not related to uremia per se, but to parathyroid proliferation in a study of phosphate-induced hyperparathyroidism concurrent with 5/6 nephrectomy (13). This may explain why EC Ca^{2+} effects on cell number are only observed at high densities presumably representing quiescent and possibly apoptotic populations, but not at lower densities which presumably represent populations in the linear or exponential proliferative phase.

We also examined regulation of parathyroid cell proliferation by $1,25\text{-}(OH)_2D_3$ and the $1,25VDR_{nuc}$ agonist, BT. $1,25\text{-}(OH)_2D_3$ is known for its anti-proliferative effects on parathyroid cells, however in our system, $1,25\text{-}(OH)_2D_3$ was associated with increased parathyroid cell viability after only a 4 hour exposure in high density culture conditions for both SHR and WKY PTC, as well as in intermediate density culture conditions for SHR PTC. In contrast, $1,25\text{-}(OH)_2D_3$ enhanced viability after 48 hours in low density culture conditions for SHR PTC and for all density conditions for WKY PTC. These results suggest that $1,25\text{-}(OH)_2D_3$ may increase cell viability by prevention of overproliferation and thus high-density induced apoptosis. This would explain why the SHR PTC, which display a faster growth rate, may be affected at both intermediate and high densities at 4 hours whereas WKY PTC are most affected after 48 hours. It would also account for the observation that only the low density group was affected after 48 hours for SHR PTC since the higher density groups would presumably have sufficient time to overcome the anti-proliferative (and therefore anti-apoptotic) effects of $1,25\text{-}(OH)_2D_3$. When we included cytosine-arabinoside in incubation with $1,25\text{-}(OH)_2D_3$ for 48 hours, we found that the effects on cell viability were abolished indicating that cell proliferation was required for the effects of $1,25\text{-}(OH)_2D_3$. Inhibition of apoptosis related to inhibition of overproliferation has been reported before in parathyroid tissue derived from normal dogs and patients with secondary hyperparathyroidism (34).

We also addressed whether the effects of 1,25-$(OH)_2D_3$ involved the newly described membrane 1,25-$(OH)_2D_3$ receptor (1,25VDR_{mem}) (35), or the classical 1,25VDR_{nuc}. We have recently shown that 1,25VDR_{mem} is increased in SHR versus WKY parathyroid cells and may be involved in mediating the rapid effects of 1,25-$(OH)_2D_3$ on PHF secretion, whereas no difference in expression of the classical 1,25VDR_{nuc} was observed (36) (see section 5.3.3). Here we showed that the vitamin D3 analog, BT which has been shown to bind the 1,25VDR_{nuc} but be incapable of exerting the rapid effects 1,25-$(OH)_2D_3$ observed in some tissues (37), was also capable of the same 1,25-$(OH)_2D_3$ effects on viability which indicates that the effects of 1,25-$(OH)_2D_3$ are likely 1,25VDR_{nuc}–dependent.

These results, taken together, support the hypothesis that the parathyroid tissue of SHR is hyperplastic, but maintains responsiveness to classical parathyroid regulators such as EC Ca^{2+} and 1,25-$(OH)_2D_3$. They also indicate that cultured SHR and WKY parathyroid cells are an appropriate model for studying the pathophysiology of parathyroid hyperplasia in this model of hypertension. Since the PTG is the source of the PHF, the development of therapeutics which target parathyroid hyperplasia may be effective in reducing the elevated plasma PHF observed in low-renin hypertension.

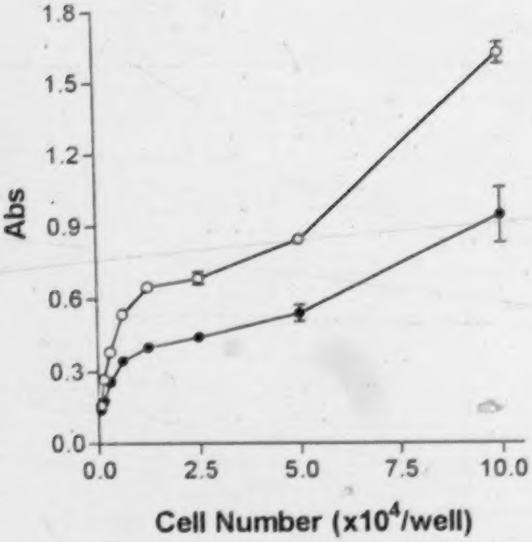


Figure 2-1—Metabolism of WST-1 by parathyroid cells derived from SHR and WKY rats. SHR (unfilled symbols) and WKY (filled symbols) parathyroid cell suspensions in Ham's F12 medium adjusted to 1.5 mM Ca^{2+} and supplemented with 5% FBS were seeded in 96-well plates at the indicated densities and the WST-1 cell proliferation assay was performed. Results are presented as the mean ± SEM of 6 replicate wells per group for one representative standard curve experiment.

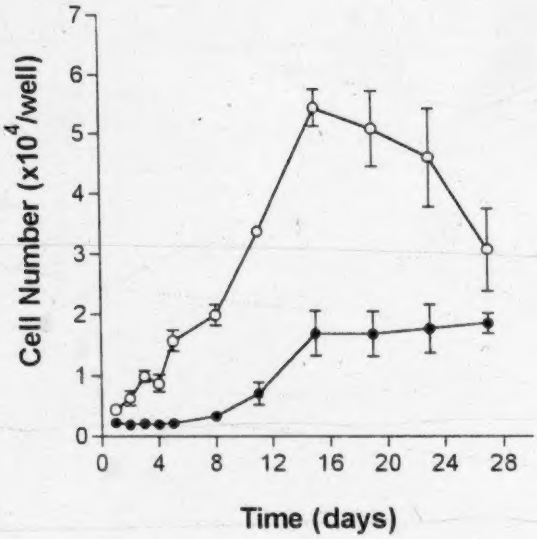


Figure 2-2— Growth curves for parathyroid cells derived from SHR and WKY rats. SHR (unfilled symbols) and WKY (filled symbols) parathyroid cell suspensions in Ham's F12 medium adjusted to 1.5 mM Ca^{2+} and supplemented with 5% FBS were seeded in 96-well plates at a density of approximately 0.2×10^4 cells/well. At the indicated time points, the WST-1 cell proliferation assay was performed. Culture medium was changed on day 4, 8, 11 and every third day after. Results are presented as the mean ± SEM of 4 experiments with 6 replicate wells per group for each experiment. Results of 2-Way ANOVA indicate that parathyroid cell number increases over time for both SHR and WKY parathyroid cell growth curves and that the two growth curves are significantly different ($P < 0.05$).

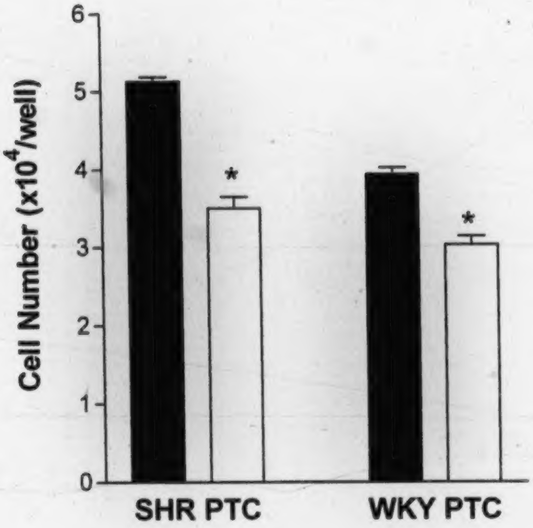


Figure 2-3—Effect of serum-deprivation on SHR and WKY parathyroid cell viability. For investigation of the effect of serum deprivation on SHR and WKY parathyroid cell viability, cells were seeded at an intermediate density on a 96-well plate. After a 48 hour attachment period, media were changed for 1.5 mM Ca^{2+} Ham's supplemented with 5% (solid bars) or 0.2% (unshaded bars) FBS for a further 96 hours. The WST-1 cell proliferation assay was then performed. *Indicates significantly decreased versus corresponding 5% FBS treatment group ($P < 0.05$; t-test).

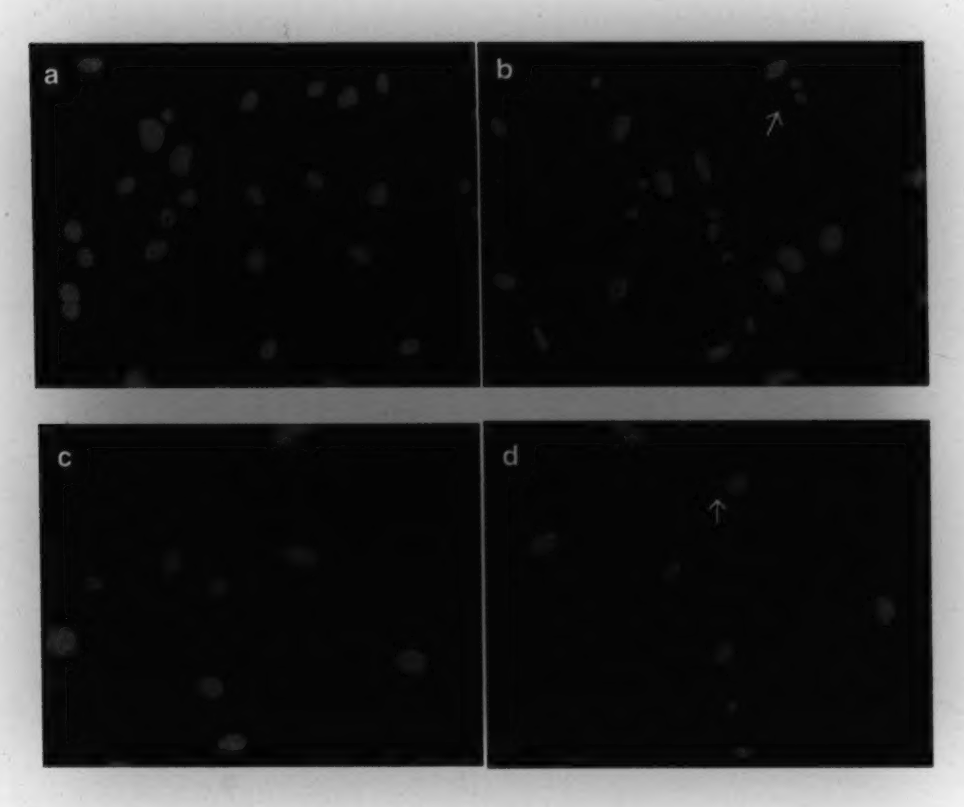
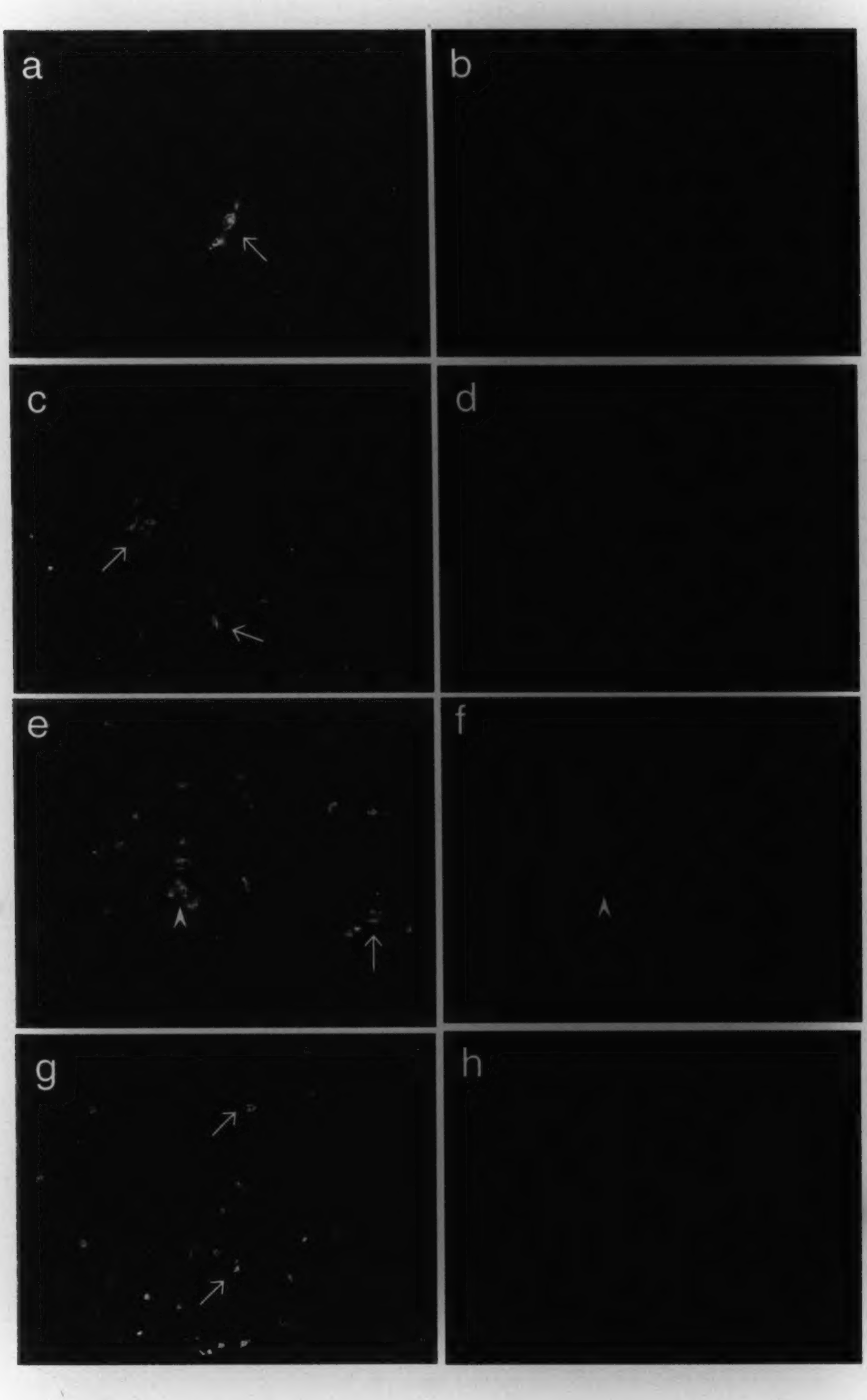


Figure 2-4—Effect of serum-deprivation on SHR and WKY parathyroid cell nuclear morphology. For investigation of the effect of serum deprivation on SHR (a, b) and WKY (c, d) nuclear morphology, cells were seeded at an intermediate density onto coverslips. After a 48 hour attachment period, media were changed for 5% (a,c) or 0.2% (b, d) FBS for a further 96 hours. Cells were stained with DAPI to visualize all nuclei. Arrows indicate fragmented or condensed nuclei.

Figure 2-5—Characterization of serum-induced SHR and WKY parathyroid cell death. To determine whether serum deprivation induced apoptosis or necrosis and to compare these processes for SHR (a-d) and WKY (e-h) parathyroid cells, cells were seeded at an intermediate density onto coverslips, allowed to attach for 48 hour and exposed to 5% (a, b, e, f) or 0.2% (c, d, g, h) FBS for a further 96 hours. Cells were stained with annexin-V-Fluos (a, c, e, g) to indicate apoptosis and propidium iodide (b, d, f, h) to visualize necrotic cells. Arrows indicate apoptotic cells and the arrowhead indicates necrotic cells.

Figure 2-5



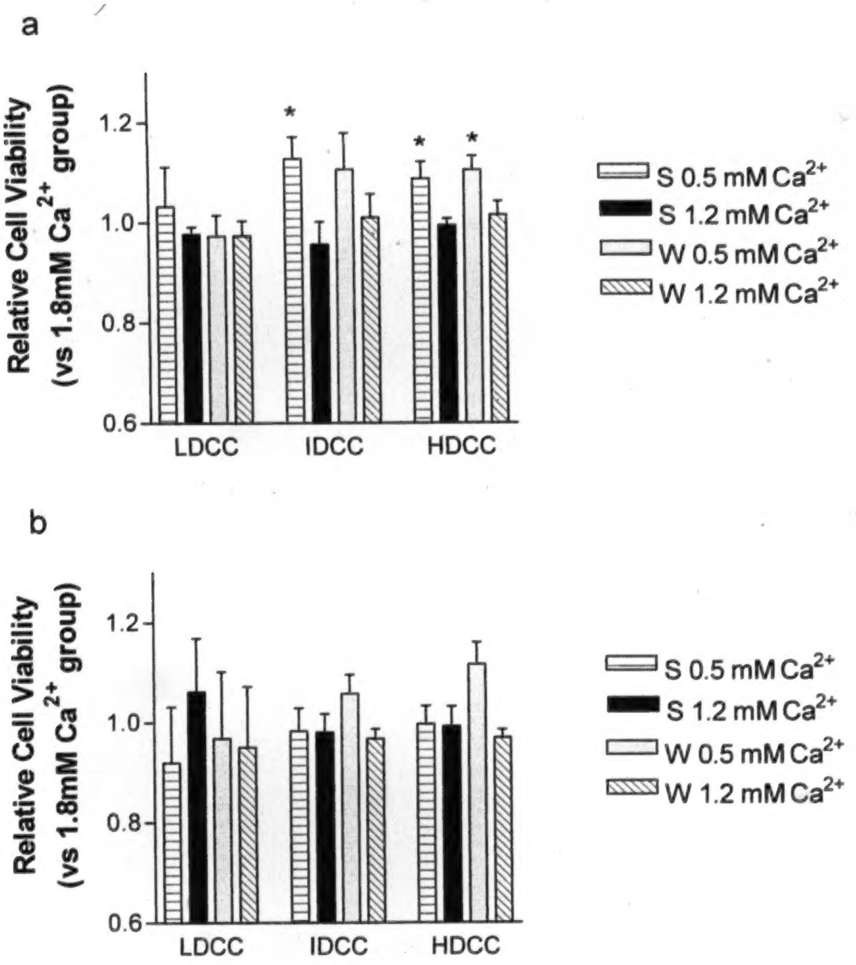


Figure 2-6—Effect of EC Ca^{2+} on SHR and WKY parathyroid cell viability. Parathyroid cells were seeded at a variety of densities to achieve the indicated cell densities at the start of the incubation period in 0.5 mM (SHR, horizontal hatched bar; WKY, dotted bar), 1.2 mM (SHR, solid bar; WKY, diagonal hatched bar), or 1.8 mM EC Ca^{2+} Ham's F12 supplemented with 5% FBS. Cells were incubated for 4 (a) or 48 (b) hours and analyzed for cell viability in the WST-1 proliferation assay. Results shown are the mean ± SEM for 4 separate experiments with a minimum of 6 wells/treatment group and are expressed relative to the 1.8 mM EC Ca^{2+} treatment group. *Indicates significantly different ($P < 0.05$; one-way ANOVA) versus 1.2 and 1.8 mM Ca^{2+} group for corresponding rat strain using actual cell numbers.

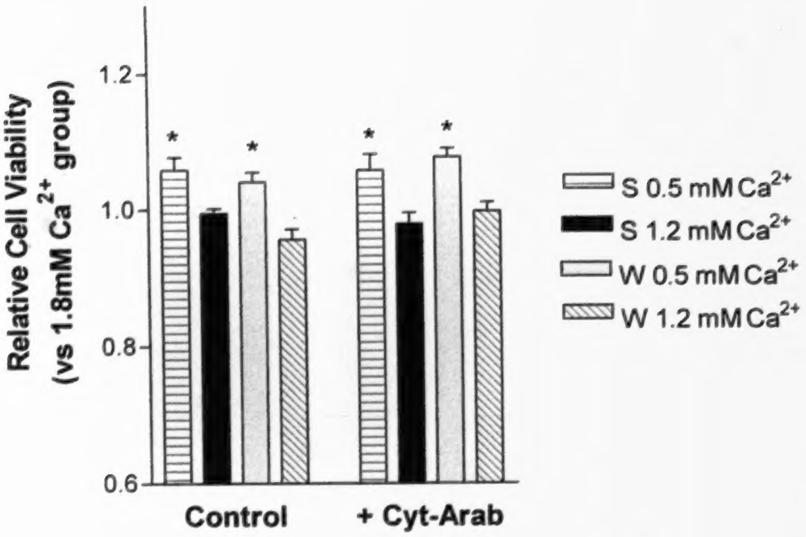


Figure 2-7—Effect of proliferation blockade on effect of EC Ca^{2+} on SHR and WKY parathyroid cell viability. Parathyroid cells at a high cell density were incubated in 0.5 mM (SHR, horizontal hatched bar; WKY, dotted bar), 1.2 mM (SHR, solid bar; WKY, diagonal hatched bar), or 1.8 mM EC Ca^{2+} Ham's F12 supplemented with 5% FBS in the presence (Cyt-Arab) or absence (Control) of 10 μM cytosine-arabinoside. Cells were incubated for 4 hours and analyzed for cell viability in the WST-1 proliferation assay. Results shown are the mean ± SEM for 4 separate experiments with a minimum of 6 wells/treatment group and are expressed relative to the appropriate 1.8 mM EC Ca^{2+} group. *Indicates significantly different ($P < 0.05$; one-way ANOVA) versus 1.2 and 1.8 mM Ca^{2+} group for corresponding rat strain using actual cell numbers.

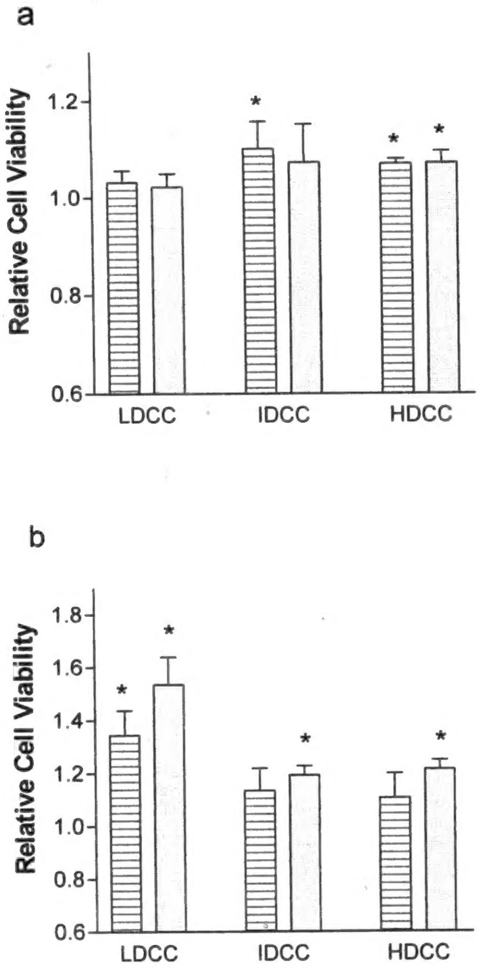


Figure 2-8—Effect of 1,25-$(OH)_2D_3$ on SHR and WKY parathyroid cell viability. Parathyroid cells were seeded at a variety of densities to achieve the indicated cell densities at the start of the incubation period in 1.5 mM EC Ca^{2+} Ham's F12 supplemented with 5% FBS containing ethanol vehicle (control) or 100 nM 1,25-$(OH)_2D_3$. SHR (horizontal hatched bars) and WKY (dotted bars) parathyroid cells were incubated for 4 (a) or 48 (b) hours and analyzed for cell viability in the WST-1 proliferation assay. Results shown are the mean ± SEM for 4 separate experiments with a minimum of 6 wells/treatment group and are expressed relative to the control group. *Indicates significantly different ($P < 0.05$; t-test) versus control group for corresponding rat strain determined using actual cell numbers.

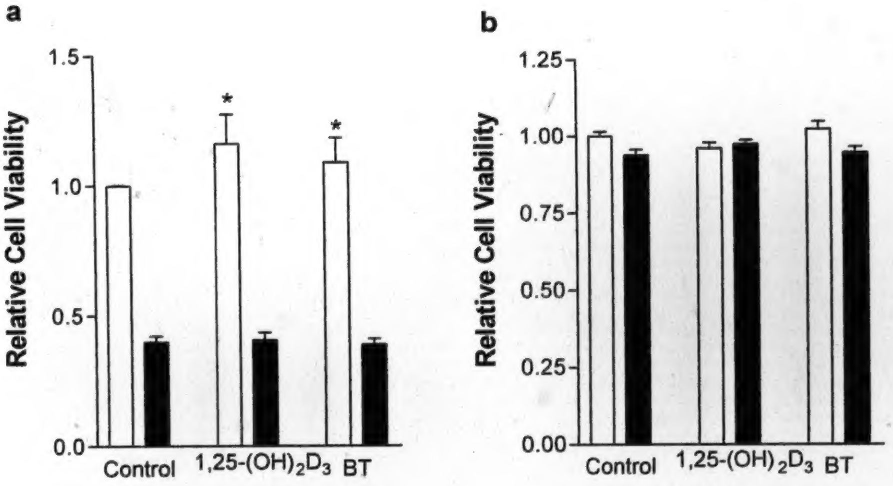


Figure 2-9—Effect of proliferation blockade on effect of 1,25-$(OH)_2D_3$ on SHR and WKY parathyroid cell viability. SHR (a) and WKY (b) parathyroid cells at a low cell density were incubated with 100 nM 1,25-$(OH)_2D_3$, analog BT, or ethanol vehicle (control) in 1.5 mM EC Ca^{2+} Ham's F12 supplemented with 5% FBS in the presence (solid bars) or absence (unshaded bars) of 10 μM cytosine-arabinoside. Cells were incubated for 48 hours and analyzed for cell viability in the WST-1 proliferation assay. Results shown are the mean ± SEM for 4 separate experiments with a minimum of 6 wells/treatment group and are expressed relative to the control group without cytosine-arabinoside. *Indicates significantly different ($P < 0.05$; t-test) versus control group determined using actual cell numbers.

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Chapter 3

Basal secretion of parathyroid hypertensive factor and immunolocalization in parathyroid cells[1]

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[1] **Portions of this chapter have been submitted to Endocrinology for publication as: Sutherland, S. K.** and C. G. Benishin. Regulation of parathyroid hypertensive factor secretion by extracellular calcium in parathyroid cells derived from spontaneously hypertensive rats.

3 BASAL SECRETION OF PHF AND PARATHYROID IMMUNOLOCALIZATION

3.1 Introduction

PHF was shown to be elevated in the plasma of SHR (1), and in low-renin rat models such hypertensive salt-sensitive Dahl rats and DOCA-NaCl hypertensive rats (2, 3) as well as in low-renin hypertensive humans (4). In addition, release of PHF from intact parathyroid organ cultures was detected in cultures derived from SHR, but not the normotensive genetic control WKY rats (5). Parathyroid cell culture experiments also suggested increased PHF release by cells derived from SHR (unpublished observations) versus WKY. However, since these experiments were designed primarily for the purpose of purifying large quantities of PHF rather than comparing production between the two strains, variables such as cell number in culture were not held constant. This makes it somewhat difficult to draw conclusions regarding differential basal PHF secretion between SHR and WKY cultured parathyroid cells. As well, the PHF blood pressure bioassay used as a PHF detection method, in addition to being cumbersome to perform, displayed significant variation and thus was only appropriate for measuring relatively large differences in PHF secretion by parathyroid cells. The recent development of both polyclonal (6) and monoclonal (7) anti-PHF antibodies has allowed for PHF detection using immunochemical methods, such as enzyme-linked immunosorbant assay (ELISA) and immunocytochemistry. The development of these techniques has simplified the detection and quantification of PHF and has improved the reliability and sensitivity of PHF measurement in secretion studies. To test the hypothesis that SHR parathyroid cells produce PHF at a greater basal rate than WKY parathyroid cells, both immunocytochemistry and the PHF ELISA were utilized.

3.2 Materials & Methods

3.2.1 Materials

Mouse anti-PHF polyclonal antibody (IgM-3A), standard semi-pure PHF, PHF-horseradish peroxidase conjugate (PHF-HRP) and mouse monoclonal anti-PHF antibody (B2) was prepared as described previously (6, 7). Vectashield was obtained from Vector Laboratories (Burlingame, CA, USA). All other chemicals were purchased from either Sigma/Aldrich (St. Louis, MO, USA) or Fisher chemicals (Fairlawn, NJ, USA). SHR and WKY rats were obtained from Charles River (St. Constant, Quebec, Canada).

3.2.2 Detection of PHF in Culture Medium and Cross-reactivity Studies

A competitive ELISA for PHF quantification in human plasma (6) has recently been developed and was used to detect PHF in parathyroid cell culture media with some modifications. Polyclonal anti-PHF antibody (IgM-3A)-coated 96-well microtiter plates (MTP) (1-4 μg/ml in 0.15 M potassium phosphate buffer; 100 μl/well) were incubated overnight at 4 ºC, followed by 2 hours at 37 ºC. Samples were either collected (20 μl/well) and pipetted directly into a blank MTP or collected on ice and frozen at -70 ºC until ready for analysis in ELISA. PHF standard in blank cell culture medium (Ham's F12 medium containing various concentrations of Ca^{2+}, $1,25\text{-}(OH)_2D_3$, or ethanol vehicle depending on the experiment, supplemented with 5% FBS) and unknown samples were diluted 1:20 in PBS (pH 7.4) and added in duplicate or triplicate wells (50 μl/well) to washed IgM-3A-coated MTP followed by addition of 50 μl/well of PHF-HRP in PBS containing 1% BSA and 0.05% Tween 20 (at various dilutions ranging from 1:4000 to 1:50 000 depending on PHF-HRP conjugate lot used) and incubated for 2 hours at 37 °C. The chromogenic peroxidase substrate tetramethyl benzidine (Sigma) was prepared according to manufacturer's specifications, added to washed ELISA MTP (100 μl/well), and incubated in darkness for 30 minutes on a shaker. The reaction was stopped by addition of 2M H_2SO_4 (50 μl/well) and absorbance of oxidized substrate read at 450 nm using a Labsystem Multiskan MCC microplate reader.

A variety of substances were tested in order to demonstrate the specificity of the monoclonal anti-PHF antibodies. The method of competitive ELISA described above was adapted for the cross-reactivity study, with minor changes in the second step of the assay. During the second step, triplicates of each PHF standard in PBS or tested analyte (50 μl/well) were pipetted and PHF-HRP conjugate (50 μl/well; 1:4000 dilution with the incubation buffer) was added and the mixture was incubated for 2 hours at 37 C. The other steps of the ELISA were similar to those described above. The results of the ELISA were calculated using the program Microsoft Excel. The percentage of inhibition was calculated as the following:

$$\%\ \text{inhibition} = 100-(A-A^b)/(A_0-A_0^b)\text{x}100$$

where A is the mean of the signal corresponding to tested analyte on the plate with monoclonal antibody, A_0 is the mean of the signal corresponding to negative control (PBS) on the plate with monoclonal antibody, A^b is the mean of the signal corresponding to tested analyte on the plate without monoclonal antibody (background), A_0^b is the mean of the signal corresponding to the negative control (PBS) on the plate without monoclonal antibody (background). The concentration of PHF corresponding to the response of the highest concentration of cross-reactive analyte used in ELISA was calculated using Microplate Manager/PC software (Bio-Rad Laboratories, Mississauga, ON, Canada). The percentage of cross-reactivity was reported as the ratios of the concentration of the analyzed cross-reactive substance yielding the same response in ELISA multiplied by 100.

3.2.3 Immunocytochemistry for PHF in parathyroid cells

Parathyroid cell cultures were obtained as described previously (8). Cultured parathyroid cells were re-suspended in DMEM containing 10% FBS and seeded onto sterile glass coverslips at 1 x 10^4 cells/coverslip and allowed a 48 hour attachment period at 37 °C in an atmosphere of 5% CO_2. Cultures were then washed once with room temperature PBS (pH 7.4) and fixed with 4% paraformaldehyde containing 0.5% Tween-20 (to allow permeabilization of antibodies) for 10 minutes. Fixed cultures were then immediately washed in PBS 3 times for 10 minutes to remove residual fixative. All subsequent incubations with reagents were performed in a humidified chamber. Non-specific antibody binding sites were then blocked with 4% BSA in PBS for 1 hour at 37 °C. Anti-PHF monoclonal antibody B2 (100 μg/ml), alone or pre-absorbed with partially purified PHF, or PBS alone were then incubated with the cells overnight at 4 °C. Cultures were washed 3 times 5 minutes in PBS and exposed to 1:100 anti-mouse IgM-FITC for 30 minutes at 37 °C in darkness. Cultures were again washed 3 times 5 minutes in PBS and mounted on slides using Vectashield mounting medium. Slides were photographed using an Olympus BX-40 fluorescence microscope and images obtained with a SPOT-2 digital camera and images were processed using Microsoft PictureIt.

3.3 Results

3.3.1 ELISA for PHF in Culture Media

PHF secretion studies necessitated the development of an ELISA for PHF detection in cell culture media. This was accomplished through modification of an existing ELISA for PHF detection in human plasma (6). These modifications were required in order to detect very low concentrations of PHF and allow determination of basal PHF secretion rates for time periods as short as 10 minutes. Many substances found in normal human plasma and known vasoactive materials were tested for cross-reactivity with the monoclonal antibody at a variety of concentrations. The results are shown in Table 3-1 and indicate that no cross-reactivity was observed with any of the substances tested (Krylova, unpublished results) (6). Figure 3-1 demonstrates the absorbance-suppressing effects of 1.5 mM Ca^{2+} Ham's F12 culture medium containing 5% FBS and typical PHF standard curves. For experiments testing the effect of EC Ca^{2+}, standard curves were initially constructed using both low (0.5 mM) and normal (1.5 mM) Ca^{2+} Ham's F12 culture medium containing 5% FBS and samples compared to the appropriate standard curve. A similar protocol was performed when testing the effect of 1,25-$(OH)_2D_3$ on PHF release. This was done in order to determine whether Ca^{2+} or 1,25-$(OH)_2D_3$ contained in the samples was capable of shifting the PHF standard curve resulting in false PHF determinations. Figures 3-2 and 3-3 show that the standard curves were not significantly different for the different concentrations of EC Ca^{2+} or the high (1 μM) concentration of 1,25-$(OH)_2D_3$ respectively. As well, in order to determine an optimal concentration of PHF-HRP to use in ELISA, various dilutions ranging from 1:4000 to 1:50 000 (depending on the lot of PHF-HRP) were tested. A negative relationship between PHF-HRP dilution and optical density is shown in Fig. 3-4. For most of the PHF secretion studies described in this thesis, PHF-HRP was used at either 1:8000 or 1:50 000 for two different conjugate lots. The limit of detection for this assay was 0.002 Units of PHF/ml and intra- and inter- assay coefficients of variance were 8.2 and 13 % respectively.

3.3.2 Basal Secretion of PHF

Using this assay, PHF release was consistently shown to be increased in parathyroid cells derived from SHR versus WKY rats. Typical results of cumulative PHF release into 1.5 mM Ca^{2+} Ham's F12 culture medium supplemented with 5% FBS after 72 hours are shown in Figure 3-5. The PHF secretion shown here is from parathyroid cell cultures that are in the plateau phase of cell growth for both SHR and WKY cells. This represents the most stable/quiescent period, therefore was deemed the most appropriate for comparison between SHR and WKY parathyroid cell cultures.

3.3.3 Immunocytochemistry for PHF in parathyroid cells

The immunocytochemical results for PHF in SHR and WKY parathyroid cells are shown in Figure 3-6. Cytoplasmic staining was observed using monoclonal anti-PHF antibody B2 for SHR (Fig. 3-6a), and to a lesser extent, WKY (Fig. 3-6b) parathyroid cells. Minimal background staining was seen in cells exposed to PBS alone (Fig. 3-6e and Fig. 3-6f) and a diminished signal was seen in cells exposed to antibody pre-absorbed with PHF (Fig. 3-6c and Fig. 3-6d).

3.4 Discussion

These results indicate that the ELISA for detection of PHF in culture medium is a specific and sensitive method for quantifying PHF secretion by parathyroid cells. Using this ELISA, secretion of PHF was shown to be enhanced in SHR cultured parathyroid cells under basal conditions. As well, the immunocytochemical staining for PHF in SHR parathyroid cells appears to be increased versus WKY parathyroid cells. This may partially account for the elevated plasma PHF observed in SHR (1). The stimulus for increased basal production in sub-cultured SHR parathyroid cells is unknown but may indicate that SHR cells have undergone a transformation to a phenotype with an increased basal synthesis and release of PHF. If novel cells, as suggested previously (9), are indeed responsible for the excess secretion of PHF in the SHR, it is possible that they may be the major cell type represented in parathyroid sub-cultures. These data provide specific evidence of increased PHF storage and secretion in parathyroid cell cultures derived from the SHR versus WKY rats.

Substance	C40D21B2
5-hydroxytryptamine	n.d.
Acetyl coenzyme A	n.d.
Adenosine triphosphate	n.d.
Alpha-melanocyte-stimulating hormone	n.d.
Angiotensin I	n.d.
Angiotensin II	n.d.
Bradykinin	n.d.
Calcitonin (salmon)	n.d.
Calcitonin-gene-related peptide	n.d.
Calcitriol	n.d.
Cholecystokinin	n.d.
Cytochrome C	n.d.
Dopamine	n.d.
Endothelin-1	n.d.
Endothelin-3	n.d.
Estrogen	n.d.
Guanosine triphosphate	n.d.
Hemoglobin	n.d.
Human Parathyroid hormone (1-84)	n.d.
Insulin	n.d.
Insulin-like growth factor-I	n.d.
Insulin-like growth factor-II	n.d.
Lysophosphatidyl choline	n.d.
Lysophosphatidyl ethanolamine	n.d.
Lysophosphatidyl inositol	n.d.
Lysophosphatidyl serine	n.d.
Melatonin	n.d.
Nerve growth factor	n.d.
Noradrenaline	n.d.
Ouabain	n.d.
Parathyroid hormone (bovine, 1-34)	n.d.
Parathyroid hormone (rat, 1-34)	n.d.
Platelet-activating factor	n.d.
Progesterone	n.d.
Prostaglandin E_2	n.d.
Secretin	n.d.
Spermidine	n.d.
Thrombin	n.d.
Thyroglobulin	n.d.
Vasopressin	n.d.

Table 3-1—Cross-reactivity of monoclonal anti-PHF antibody B2 with various substances in the PHF ELISA. None of the indicated substances demonstrated detectable cross-reactivity with the monoclonal anti-PHF antibody B2. n.d. indicates not-detectable. (Krylova, unpublished results).

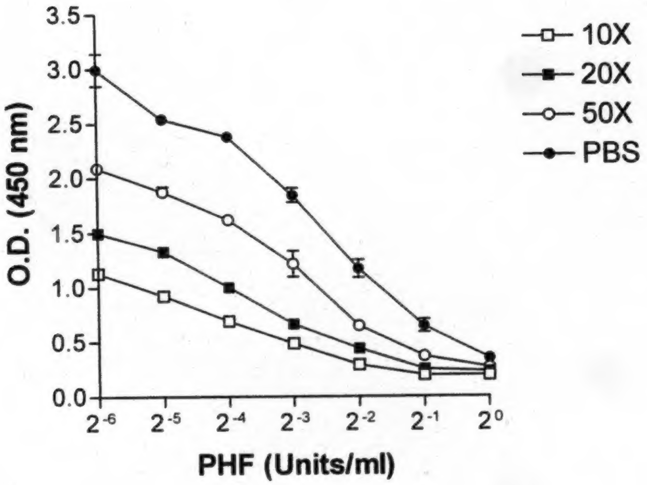

Figure 3-1—Effect of culture medium on standard curves for the ELISA for PHF detection in culture media samples. The effect of 10, 20, and 50 times (unshaded square, shaded square, and open circle symbols respectively) diluted culture medium in PBS vs PBS alone (shaded circle symbols) on the PHF standard curve final absorbance at 450 nm is shown.

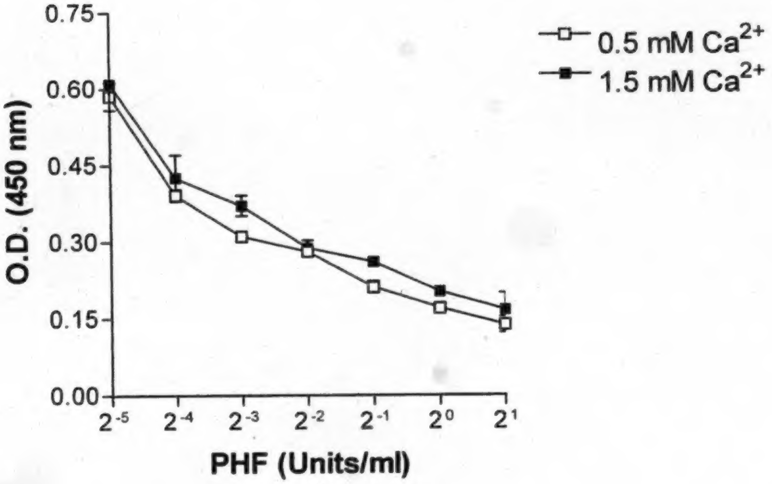

Figure 3-2—Effect of calcium concentration on standard curves for the ELISA for PHF detection in culture media samples. The effect of 0.5 and 1.5 mM Ca^{2+} (unshaded square and shaded square symbols respectively) Ham's F12 medium supplemented with 5% on the PHF standard curve final absorbance at 450 nm is shown.

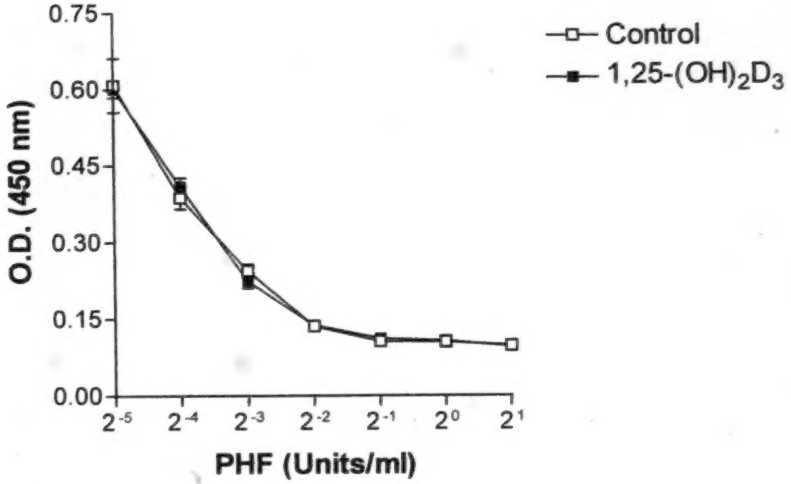

Figure 3-3—Effect of 1,25-$(OH)_2D_3$ on standard curves for the ELISA for PHF detection in culture media samples. PHF standard curves were constructed in 1.5 mM Ca^{2+} Ham's F12 medium supplemented with 5% FBS containing either 1 μM 1,25-$(OH)_2D_3$ or ethanol vehicle (shaded square and un-shaded square symbols respectively).

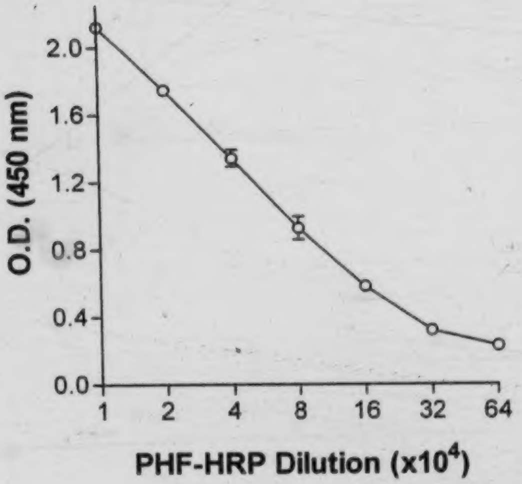

Figure 3-4—Effect of PHF-HRP conjugate dilution on final absorbance at 450 nm in ELISA for PHF detection in culture media samples. Various concentrations of PHF-HRP were incubated in ELISA with PBS containing 5% culture medium (Ham's F12 medium adjusted to 1.5 mM Ca^{2+} and supplemented with 5% FBS) and the optical density at 450 nm measured.

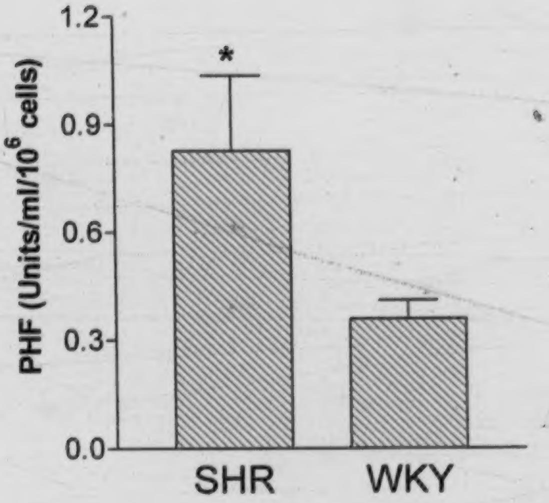

Figure 3-5—Secretion of PHF by cultured SHR and WKY parathyroid cells. Cumulative PHF release into 1.5 mM Ca^{2+} Ham's F12 culture medium (supplemented with 5% FBS) after 72 hours by cultured SHR and WKY parathyroid cells was measured by ELISA. Results are presented as mean ± SEM PHF values for 3 separate experiments with 6 culture dishes for each cell type. *Indicates significantly increased versus WKY parathyroid cell cultures ($P < 0.05$; t-test)

6

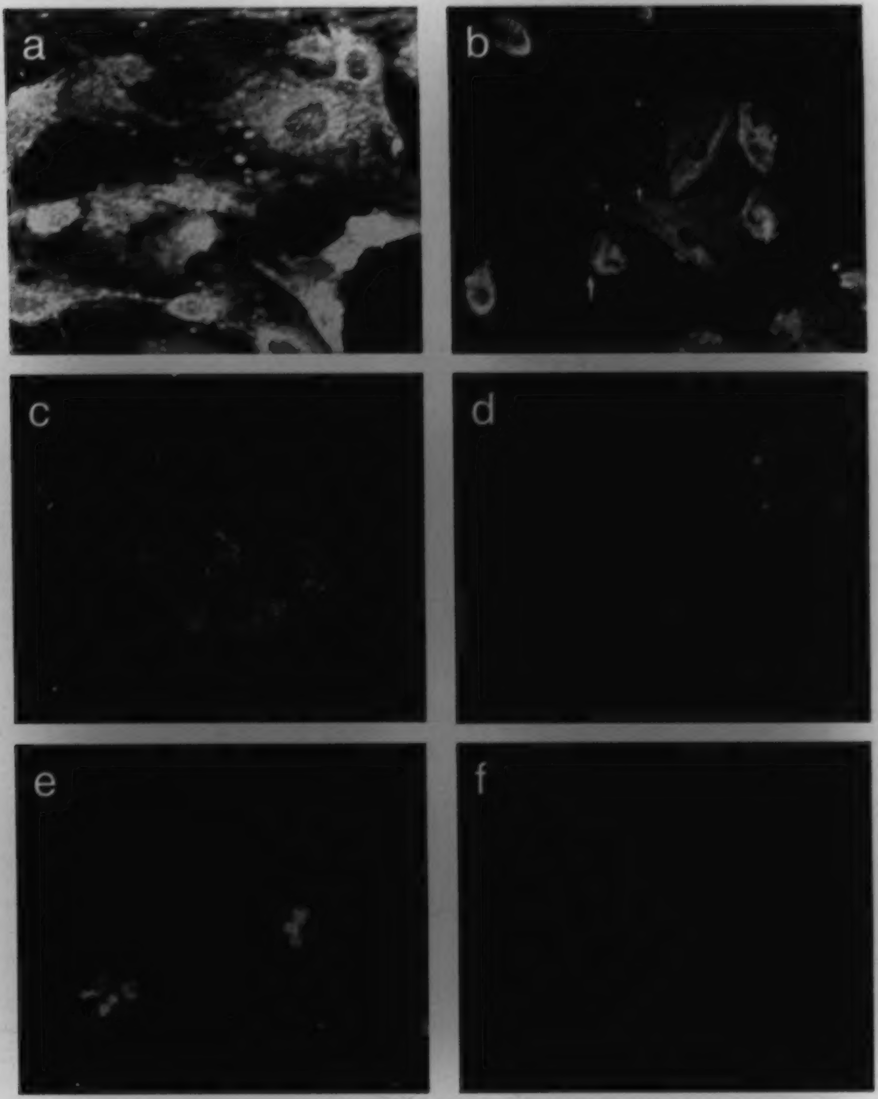

Figure 3-6—Immunolocalization of PHF in cultured SHR and WKY parathyroid cells. Cultured SHR (a,c,e) and WKY (b,d,f) parathyroid cells were incubated with monoclonal anti-PHF antibody B2 (a,b), B2 pre-absorbed with PHF (c,d), and PBS alone (e,f).

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Chapter 4

Regulation of parathyroid hypertensive factor secretion by extracellular calcium in parathyroid cells derived from spontaneously hypertensive rats[1]

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4 EFFECT OF EC Ca^{2+} ON PHF SECRETION

4.1 Introduction

Extracellular ionized calcium (EC Ca^{2+}) regulates the activity of many diverse cell types including skeletal, cardiac, and vascular smooth muscle cells; neurons; endocrine and exocrine secretory cells; and many others (1). In most systems EC Ca^{2+} stimulates secretory activity, however, due to the physiological role of the PTG in maintaining plasma calcium homeostasis, EC Ca^{2+} has atypical effects on the parathyroid cell (2). The PTG secrete PTH in response to low serum ionized calcium which then acts upon the classical PTH target sites, the kidneys and bone, to counteract the fall in plasma Ca^{2+}. The endocrine axis is completed with negative feedback effects of calcium on the parathyroid including inhibition of PTH synthesis and secretion (3-6) as well as effects on proliferation (7, 8). The mechanism of action of EC Ca^{2+} in the parathyroid has been reviewed elsewhere (9), but Ca^{2+} appears to act as a first messenger upon the G-protein-coupled CaR (10).

Abnormalities in parathyroid function, including hyperparathyroidism, may be associated with conditions not typically involved in calcium homeostasis. One such condition is hypertension. Furthermore, when hypertensive patients are classified according to plasma renin activity, the low-renin subset (representing approximately 30-40%) exhibits a plasma calcium deficit accompanied by appropriate shifting of the calcium-regulating hormones resulting in increased plasma PTH, elevated 1,25-dihydroxyvitamin D_3, and suppressed calcitonin (11). In addition to the association of hyperparathyroidism with low-renin hypertension, parathyroidectomy (12, 13) and parathyroid cross-transplantation studies in SHR have demonstrated parathyroid involvement in hypertension (12). Several dietary studies have also addressed the role of calcium balance in hypertension, but the results have been inconsistent and confusing. However, when plasma renin profile is taken into account, a high calcium diet has been shown to ameliorate hypertension in both low-renin hypertensive rat models (14, 15) and human hypertension (11, 16, 17). Surprisingly, Ca^{2+} channel blockers have also exhibited anti-hypertensive effects in low-renin hypertension (18). This is a paradox since one treatment elevates Ca^{2+} availability to cells, but the other prevents its entry into cells.

This paradox may be resolved by the Parathyroid Hypertensive Factor (PHF) (for review see (19)). PHF was originally discovered and isolated from the plasma of SHR (20) and later found to be elevated in other low-renin rat models of hypertension (21, 22), as well as in primary hyperparathyroid (13) and low-renin hypertensive humans (23). Subsequently, lower levels of PHF have been detected in plasma of normotensive humans (unpublished observations). The hypertensive mechanism of action of PHF involves the potentiation of the effects of classical vasoconstrictors by increasing Ca^{2+} influx into vascular smooth muscle cells through L-type Ca^{2+} channels thereby accounting for the efficacy of Ca^{2+} channel blockers (24). The beneficial effects of dietary calcium may then be explained by the fact that the major source of PHF is the PTG (12, 25, 26), a tissue which is negatively regulated by plasma Ca^{2+}.

The regulation of PHF synthesis and secretion is poorly understood, however initial studies have indicated that higher levels of EC Ca^{2+}, as for PTH, appear to suppress PHF release. Increased dietary calcium intake was associated with lower PHF levels in both low-renin rat models (27, 28) and human hypertensives (29) and a reduction in culture media Ca^{2+} concentration enhanced PHF detected by the blood pressure bioassay in parathyroid organ cultures derived from SHR (25). Here we have investigated the effects of EC Ca^{2+} on PHF secretion in low-renin hypertension and normotension by utilizing a continuous SHR and WKY parathyroid cell culture system (26). As well, the role of the CaR in mediating the effects of EC Ca^{2+} on PHF release was also examined.

4.2 Materials & Methods

4.2.1 Materials

Mouse anti-PHF polyclonal antibody (IgM-3A), standard semi-pure PHF, and PHF-horseradish peroxidase conjugate (PHF-HRP) were prepared as described previously (30). Polyclonal rabbit anti-CaR IgG (PA1-934) and control CaR neutralizing peptide (PA1-934) were purchased from Affinity Bioreagents (Golden, CO, USA). All cell culture media were obtained from Gibco/BRL (Burlington, ON). Acrylamide bis, temed, ammonium persulfate, and kaleidoscope molecular weight standards were all purchased from BioRad (Hercules, CA, USA). Enhanced chemiluminescence (ECL) detection reagents for Western blot analysis were obtained from Amersham (Arlington Heights, IL, USA). WST-1 cell proliferation reagent was obtained from Roche Biochemicals (Mannheim, Germany). All other chemicals were purchased from either Sigma/Aldrich (St. Louis, MO, USA) or Fisher chemicals (Fairlawn, NJ, USA). SHR and WKY rats were obtained from Charles River (St. Constant, Quebec, Canada).

4.2.2 Detection of PHF in Culture Medium

PHF detection in culture medium was performed as described in section 3.2.2.

4.2.3 Effect of EC Ca^{2+} on PHF Secretion

Continuous parathyroid cell cultures were established as described previously (26). Cultured parathyroid cells were seeded and allowed a 48 hour attachment period in DMEM containing 10% FBS and then exposed to Ham's F12 medium adjusted with $CaCl_2$ to contain various concentrations of Ca^{2+} and supplemented with 5% FBS for the indicated time periods. Aliquots were removed and processed for PHF analysis in ELISA as indicated above and the cells processed for the WST-1 cell viability assay. For experiments involving parathyroid organ culture, glands were isolated from SHR and WKY rats as described previously (26) and placed in 24-well culture dishes (2 glands/well) containing 250 μl/well Ham's F12 medium adjusted to contain either low (0.5 mM) or normal (1.5 mM) Ca^{2+} and supplemented with 5% FBS. Aliquots of 10 μl were removed at various time points for PHF determination as described above. Use of animals was according to CCAC guidelines, and reviewed and approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

4.2.4 Determination of Parathyroid Cell Number

Parathyroid cell number was determined by using either the trypan blue exclusion method or the WST-1 cell proliferation assay. For cell number determination on 96-well culture plates, 10 μl WST-1 reagent was added to each well (containing 100 μl of phenol red-free 1.5 mM Ca^{2+} Ham's F12 medium + 5% FBS) and the plates incubated 2 hours at 37 °C, 5% CO_2. Optical density at 450 nm (with reference wavelength of 650 nm) was then measured by a 96-well spectrophotometer. Absorbance was compared to a cell standard curve prepared just prior to addition of WST-1, with cell number determined by the trypan blue exclusion method. For all other experiments, parathyroid cell suspensions were obtained by trypsin treatment and cell viability determined using the trypan blue exclusion method.

4.2.5 Western Blots for CaR protein

Preparation of parathyroid cell lysates and detection of the CaR protein was performed as previously described (31) with a few modifications. Parathyroid cells cultured in 6-well plates were washed twice with PBS containing 1 mM EDTA and a lysis buffer (10 mM Tris-HCl, 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1% SDS, pH 7.4) pre-heated to 65 °C was added. The cell lysate was harvested and incubated for 5 minutes at 65 °C and aliquots were frozen at -70 °C until Western blot analysis. Protein content of the samples was measured using the BCA method (Pierce Chemical Company) and samples were diluted 1:1 in non-reducing SDS-Laemmli sample buffer and 5 μg electrophoresed by SDS-PAGE on 7 or 8% polyacrylamide gels. Proteins were transferred to a PVDF membrane by electroblotting at 75 V for 1 hour in transfer buffer (19 mM Tris-HCl, 150 mM glycine, 20% methanol) and the membrane equilibrated in Tris-buffered saline + 1% Tween-20 (TBST). Non-specific binding sites were blocked with 5% skim milk in TBST for 2 hours at room temperature followed by incubation with 1:1000 rabbit polyclonal anti-CaR IgG overnight at 4 °C. Blots were then washed (three times 10 minutes in TBST) and incubated with 1:2000 goat anti-rabbit IgG-HRP in TBST (30 minutes in darkness at room temperature). Membranes were washed (three times 10 minutes in TBST) and the specific proteins visualized using the enhanced chemiluminescence (ECL) detection system.

4.2.6 Role of the CaR in effects of EC Ca^{2+} on PHF release

SHR parathyroid cells were seeded at 0.5 x 10^4 cells/well and WKY cells were seeded at 1.0 x 10^4 cells/well in 96-well plates and allowed to attach/grow in 5% FBS DMEM for 48 hours. This was done to compensate for different growth rates of cells from the two strains such that approximately the same density for both strains existed prior to initiation of the experiment. Cells were incubated with PBS containing BSA (1.4 μg/ml) primary anti-CaR antibodies (raised against a control CaR peptide of the N-terminus of the CaR) alone (1.4 μg/ml) or previously pre-absorbed (1 hour, 37 °C; equal concentrations by weight of antibody and control CaR peptide) anti-CaR antibody were incubated on cells for 60 minutes at 37 °C and 5% CO_2. Cells were washed with HBSS and media replaced with either low (0.5) or normal (1.5) mM Ca^{2+} Ham's F12 supplemented with 5% FBS for 60 minutes and samples collected for PHF analysis in ELISA.

4.2.7 Statistical Analysis

ELISA standard curves and unknown values were determined using the Microplate Manager/PC software (BioRad Laboratories). Results are expressed as the mean ± SEM and statistical evaluation for differences between treatment groups was performed using either t-tests or ANOVA where appropriate.

4.3 Results

4.3.1 Effect of EC Ca^{2+} on PHF Secretion

Experiments on both SHR and WKY parathyroid cells were initially performed at similar cell densities, with the assumption that PHF release would be standardized by expressing PHF/cell. However, subsequent experiments demonstrated that the extent of the effect of calcium on parathyroid cells varied greatly with cell density in culture. Therefore, the effect of EC Ca^{2+} on rate of PHF secretion was examined at various time points after seeding SHR and WKY parathyroid cells at a low cell density. Experiments were performed over a three week period thus allowing a wide range of cell densities to be examined. During the early phase of cell growth the rate of PHF secretion decreased and there was no effect of low EC Ca^{2+} on PHF release. However, by 8 days for SHR parathyroid cells (Figure 4-1a) and 15 days for WKY parathyroid cells (Figure 4-1b), low EC Ca^{2+} had a significant and rapid stimulatory effect PHF release. Interestingly, these two time points for SHR and WKY parathyroid cells represent similar densities (~2 x 10^4 cells/well). PHF secretory rate rapidly declined and the effect of EC Ca^{2+} on PHF release disappeared when cells became quiescent or began to die after approximately 3 weeks in culture. Taken together, these results suggest that regulation of PHF release by EC Ca^{2+} has either a time or cell culture density dependency or combination of both.

To examine whether cells exposed to low EC Ca^{2+} could be further stimulated to secrete PHF after treatment periods longer than 60 minutes, cumulative PHF release after 4, 24 and 48 hours was examined. When parathyroid cells at an intermediate density (representing the same density in which low EC Ca^{2+} stimulated PHF release in the above experiments), were exposed to either low (0.5 mM) or normal (1.5 mM) EC Ca^{2+} for 4-48 hours, cells were initially stimulated to secrete PHF in the low (0.5 mM) treatment group

(45% increase versus the 1.5 mM Ca^{2+} group, $P < 0.05$ after 4 hours) however the effect disappeared after 24 hours of stimulation in SHR parathyroid cell cultures (Figure 4-2a). Similar results were observed for WKY parathyroid cell cultures (66% increase versus the 1.5 mM Ca^{2+} group, $P < 0.05$ after 4 hours) (Figure 4-2b). Interestingly, PHF in the 0.5 mM EC Ca^{2+} group detected at 48 hours was significantly decreased versus 24 and 4 hour PHF release in WKY parathyroid cell cultures. This trend was also observed in SHR parathyroid cell cultures, but was not significant ($P = 0.07$). These results suggest a decrease in sensitivity to EC Ca^{2+} after exposure times greater than 48 hours and a possible degradation/internalization of secreted PHF by parathyroid cells under low EC Ca^{2+} conditions.

In order to establish the validity of our parathyroid cell culture model in studying the mechanisms controlling PHF release, the effect of EC Ca^{2+} on PHF release was examined in cultured intact PTG derived from both SHR and WKY parathyroid cells (Figure 4-3). Intact PTG in culture displayed similar regulation by Ca^{2+}; low EC Ca^{2+} stimulated PHF secretion from both SHR and WKY PTG and, as seen in parathyroid cell culture, the effect of calcium disappeared after 24 hours. SHR PTG also displayed enhanced secretion of PHF in response to low Ca^{2+} versus WKY parathyroids at both the 4 hour (66.8-fold versus 23.9-fold increase above basal) and 24 hour (5.39-fold versus 2.46-fold increase above basal). The stimulation of PHF release by low EC Ca^{2+} was also substantially greater for intact PTG than for sub-cultured parathyroid cells for both SHR and WKY cultures.

4.3.2 Regulation of CaR protein expression

Reports of downregulation of the CaR in cultured bovine parathyroid cells (32) and the observation that sensitivity of PHF secretion to EC Ca^{2+} occurred only after shorter exposure times or higher cell densities in our own model prompted the examination of expression of the CaR under various experimental conditions. Figure 4-4 shows a 121 and 128 kDa doublet presumably representing the unglycosylated monomeric form of the CaR, a 160 kDa form which likely represents the glycosylated monomeric form of the CaR, several higher MW bands greater than 210 kDa representing CaR dimers (33), and several low MW fragments that are likely degraded CaR fragments. The band greater than 210 kDa is consistently increased in SHR vs WKY parathyroid cells that have been cultured at the same cell density. Figure 4-5 shows that CaR protein (the 121 and 128 kDa doublet) increases in both strains over time in culture and that CaR expression was also upregulated in response to 1.5 mM versus 0.5 mM EC Ca^{2+} in SHR parathyroid cells (Figure 4-5a) and this upregulation was observed by 24 hours. However in WKY parathyroid cells (Figure 4-5b), the cells exposed to 0.5 mM EC Ca^{2+} for 24 hours had increased CaR protein (indicated by increased bands greater than 210 kDa, and to a lesser extent, increased 121 and 128 kDa doublet protein) vs 1.5 mM EC Ca^{2+}. Figure 4-6 shows that higher cell density culture conditions are associated with greater expression of CaR protein (expressed relative to total cellular protein) for both SHR (Figure 4-6a) and WKY (Figure 4-6b) parathyroid cultures. Taken together, these results show that CaR expression is both EC Ca^{2+}- and cell density-regulated and is elevated in cultured SHR parathyroid cells.

4.3.3 Role of the CaR in mediating the effects of Ca^{2+} on PHF Secretion

The effect of anti-CaR IgG antibodies on PHF release under both low (0.5 mM) and normal (1.5 mM) EC Ca^{2+} is shown in Figure 4-7. Pre-incubation of anti-CaR antibodies with parathyroid cells prevented the inhibitory effect of 1.5 mM EC Ca^{2+} on PHF release from SHR parathyroid cells and restored PHF secretion to levels comparable with those observed in all 0.5 mM EC Ca^{2+} treatment groups whereas anti-CaR antibodies which had been pre-absorbed with the control CaR peptide had no effect. Anti-CaR antibodies incubated with WKY parathyroid cells however not only restored, but stimulated PHF release in the 1.5 mM EC Ca^{2+} treatment group. As well, anti-CaR antibodies prevented the stimulatory effect of low (0.5 mM) EC Ca^{2+} on PHF release. These data suggest that the CaR has an important role in mediating the effects of EC Ca^{2+} on PHF secretion.

4.4 Discussion

In this study, parathyroid cells derived from the hypertensive model SHR, which exhibits some features of secondary hyperparathyroidism, were compared to cells derived from the normotensive control strain, WKY and the effect of EC Ca^{2+} on secretion of parathyroid hypertensive factor was examined. Based on previous studies utilizing the PHF blood pressure bioassay and the observed suppressed plasma Ca^{2+} and elevated plasma PHF in the SHR, our hypothesis was that low EC Ca^{2+} would directly stimulate PHF secretion via the CaR. This study confirmed and further characterized the inhibitory effect of EC Ca^{2+} on PHF release and validated the parathyroid cell culture system for the study of PHF secretion. This is also the first report of PHF release from parathyroid cells derived from normotensive animals (WKY rats). This has the important implication that

PHF may be present physiologically. The physiological function is unclear, however it is tempting to speculate that PHF may play a role in the maintenance of intracellular Ca^{2+} in the face of suppressed plasma Ca^{2+} (19). Furthermore, the CaR was shown to be an important mediator of the effects of EC Ca^{2+} on PHF secretion and its expression was regulated by both EC Ca^{2+} and cell density in parathyroid cell culture. These data support the idea that hypocalcemia contributes to the elevated PHF status of the SHR.

Previous *in vivo* studies have demonstrated a negative relationship between plasma calcium or calcium intake and plasma PHF levels (27, 28). Furthermore, one *in vitro* study of intact PTG organ cultures showed that reduced Ca^{2+} in the culture medium resulted in enhanced PHF release (25). In these studies, PHF was measured by the only available assay at the time, a semi-quantitative blood pressure bioassay. The development of an ELISA for detection of PHF in culture media has enabled more precise studies of the direct effects of EC Ca^{2+} on PHF release including a more relevant Ca^{2+} concentration range, acute and sub-acute time points, and secretion from both PTG organ and cell culture. Modification of the existing ELISA for detection of PHF in human plasma (30) has resulted in increased sensitivity allowing for PHF detection after incubation periods as short as ten minutes.

In this study, the relationship between both basal and calcium-modulated PHF secretion rate and parathyroid cell growth was examined. The results indicated that initially, EC Ca^{2+} had no significant effect on the rate of PHF secretion. As well, PHF secretion rate during this early growth period was negatively correlated with parathyroid cell number up to approximately four days in culture for both SHR and WKY derived parathyroid cells. This is not particularly surprising since many endocrine cells, when not in the quiescent G_0 phase of the cell cycle, are not in an active secretory state. At relatively higher cell densities when cell growth was nearing a plateau however, EC Ca^{2+} had direct inhibitory effects on PHF secretion which were first apparent by 8 days for SHR and 15 days for WKY parathyroid cells. Interestingly, these two time points occur at similar densities which suggest that a critical cell density may be necessary for EC Ca^{2+} sensitivity in these cultured parathyroid cells. For both cell lines, parathyroid cell death after approximately 3 weeks in culture was accompanied by a diminished PHF secretion rate and abolished regulation by EC Ca^{2+}.

Parathyroid response to EC Ca^{2+} was also dependent on the incubation time. When both parathyroid cells and intact PTG in culture were exposed to low EC Ca^{2+} for up to 48 hours, PHF secretion was only stimulated at the 4 hour, and to a lesser extent, the 24 hour time points. This does not correlate with expression of the CaR since CaR protein was shown to increase over time in cultured parathyroid cells. The cause of this loss of sensitivity is unknown but may indicate that low EC Ca^{2+} triggers rapid release of a stored pool of PHF which is exhausted and not replaced by 48 hours. It is also possible that EC Ca^{2+} induces the release of autocrine/paracrine factors which may counteract the direct effects of EC Ca^{2+} on PHF release. Interestingly, exposure to low EC Ca^{2+} was also shown to result in reduced CaR protein expression in both SHR and WKY (after 48 hours exposure only) parathyroid cells versus the normal (1.5 mM) EC Ca^{2+} implying that reduced CaR expression in the low EC Ca^{2+} group may contribute to the loss of sensitivity with respect to PHF release. A decrease in detectable PHF in the low Ca^{2+} group by 48 hours in both the parathyroid organ and cell culture experiments was also observed suggesting that PHF is metabolized or internalized after it is released.

Although bovine parathyroid cells lose the ability to be regulated by EC Ca^{2+} (32) related to downregulation of the CaR, several other long-term parathyroid cell culture systems in addition to our own model, have successfully retained regulation by EC Ca^{2+}, and when examined, expression of the CaR (34-36). At least one other rat parathyroid culture model has been developed which retains sensitivity to EC Ca^{2+} (PT-r clonal cell line) (34), although these cells were found to secrete PTHrP rather than PTH. Here we have shown that sub-cultured primary parathyroid cells derived from both normotensive WKY rats and the low-renin hypertensive model, SHR, are regulated by EC Ca^{2+}, although to a much lesser extent than intact parathyroid organ cultures. Furthermore, sub-cultured WKY parathyroid cells, and to a greater extent SHR parathyroid cells, express several forms of the CaR protein, including the native unglycosylated monomer (approximately 120 kDa), the glycosylated monomer (approximately 160 kDa), and dimers (greater than 210 kDa) (33). Since the SHR is characterized by hypocalcemia and we have shown that EC Ca^{2+} upregulates the CaR in SHR parathyroid cells, we would expect reduced expression of the CaR. Furthermore, we have also shown that CaR expression is reduced in proliferating parathyroid cells and previous data indicate that the SHR parathyroid cells proliferate at a much faster rate than the WKY parathyroid cells (unpublished observations) which suggests that there may be some dysregulation in the expression of the CaR in SHR parathyroid tissue.

These alterations in the expression of the CaR are particularly relevant since the effect of EC Ca^{2+} on PHF secretion appears to be mediated by the CaR. In parathyroid cells derived from normotensive WKY rats, pre-incubation with anti-CaR antibodies directed against an N-terminus peptide sequence of the CaR not only restored, but stimulated PHF release in the 1.5 mM EC Ca^{2+} treatment group above and beyond the stimulation by 0.5 mM EC Ca^{2+} in the control groups. This indicates that the CaR is responsible for mediating the suppressive effects of EC Ca^{2+} on PHF release. The stimulation by anti-CaR antibodies may suggest that even low levels of EC Ca^{2+} may exert some suppression of PHF release via the CaR. Interestingly, anti-CaR antibodies also prevented the stimulatory effect of low (0.5 mM) EC Ca^{2+} on PHF release indicating that the CaR is also responsible for mediating specific stimulatory signals of low EC Ca^{2+}. This is not unusual if EC Ca^{2+}-mediated signaling is viewed as analogous to peptide-based signaling in which it is common for low doses of a peptide to activate different signaling pathways than those activated by high doses through the same receptor. Since the SHR parathyroid cells were found to express greater amounts of CaR protein and had increased responses to stimulation of PHF release by low EC Ca^{2+}, it was anticipated that the CaR would mediate both high and low EC Ca^{2+}-induced effects on PHF secretion. However, in parallel experiments performed in SHR-derived parathyroid cells, the CaR appeared to mediate the inhibitory effect of 1.5 mM EC Ca^{2+} on PHF release, but not the stimulatory effect of 0.5 mM EC Ca^{2+} on PHF release. This has the important implication that the CaR of the SHR parathyroid may be modified or alternatively, other EC Ca^{2+}-sensing receptors may be involved in regulation of PHF release.

Since the effect of EC Ca^{2+} on PHF release was shown to be cell density dependent and involved the CaR, we investigated expression of the CaR at different densities in cell culture. The pivotal role of the CaR in regulation of proliferation of parathyroid cells is demonstrated by the parathyroid hyperplasia in pathologies involving mutations in the CaR, such as familial hypercalcemia hypercalciuria (FHH), neonatal severe hyperparathyroidism (NSHPT) (37), autosomal dominant hypocalcemia (38), as well as by the CaR mouse knockout model (39). In our cell culture model, EC Ca^{2+} first exerted significant effects on the rate of PHF secretion at similar cell densities for both SHR and WKY derived parathyroid cells. This property may be explained by the observation that expression of the CaR is up-regulated under higher cell density culture conditions. At first glance, this is somewhat unexpected since several studies have shown reduced parathyroid expression of the CaR in both primary (40, 41) and secondary hyperparathyroidism (42); conditions which are characterized by parathyroid cell overproliferation. For example, Brown *et al* concluded that downregulation of the CaR is not related to uremia per se, but to parathyroid proliferation in a study of phosphate-induced hyperparathyroidism concurrent with 5/6 nephrectomy (42). However perhaps the higher cell density/plateau phase of cell growth in our culture model is not represented in the pathological hyperplastic parathyroid tissue. The hyperplasia of hyperparathyroidism is probably more accurately represented by the early, low density phase of our culture system in which CaR expression is continually reduced. This CaR downregulation could account for the lack of the effect of EC Ca^{2+} on PHF release during the early parathyroid growth phases of our models.

In conclusion, this study demonstrates that secretion of PHF by parathyroid cells derived from both SHR and WKY rats is stimulated by exposure to low EC Ca^{2+}. Furthermore, these effects were shown to be mediated by the CaR and largely dependent on cell density which may at least partially account for the strain differences in the PHF response to EC Ca^{2+}. Taken together, these results provide support for the idea that the low plasma Ca^{2+} observed in SHR may contribute to the elevated PHF secretion observed in this model.

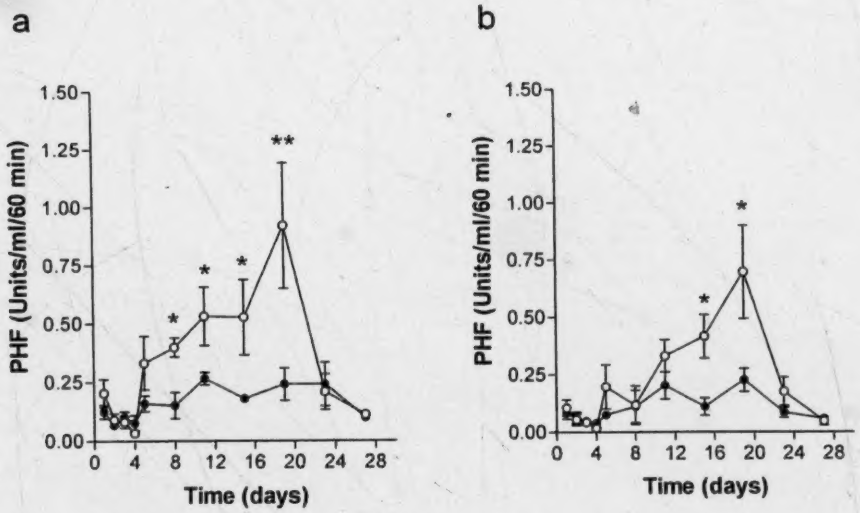

Figure 4-1—Rapid effect of extracellular Ca^{2+} on PHF release by subcultured parathyroid cells. For investigation of the effect of EC Ca^{2+} on rate of PHF release/60 minute at various growth stages of SHR (a) and WKY (b) parathyroid cells, cell suspensions in Ham's F12 medium supplemented with 5% FBS were seeded in 96-well plates at a density of 0.2×10^4 cells/well (Day 0). At the indicated time points, medium was gently aspirated and replaced with either 0.5 or 1.5 mM Ca2+ Ham's F12 medium + 5% FBS (100 µl/well) and incubated for 60 minutes at 37 °C. Aliquots of 50 µl/well were then collected and processed for PHF analysis, media aspirated and replaced with fresh 1.5 mM Ca2+ Ham's F12 medium + 5% FBS (100 µl/well) and analyzed for cell viability. Culture medium was replaced every three days with fresh growth medium. Data points represent mean PHF ± SEM of four experiments with 6 culture wells for each treatment group. The open and filled symbols indicate 0.5 or 1.5 mM Ca^{2+} groups respectively. *Value is significantly different ($P<0.05$; t-test) from 1.5 mM Ca^{2+} data point at the indicated time point.

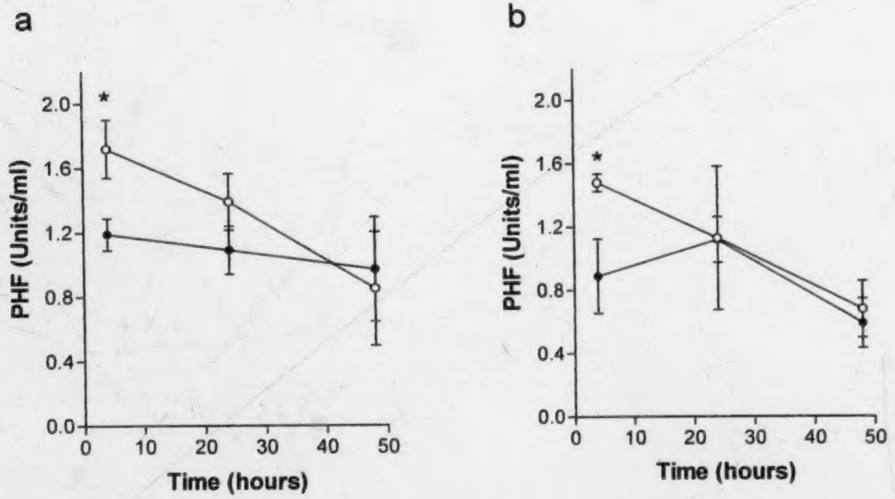

Figure 4-2—Time course for effect of extracellular Ca^{2+} on PHF release by subcultured parathyroid cells. For investigation of the sub-acute (4-48 hour) effects of EC Ca^{2+} on PHF release, parathyroid cell suspensions in DMEM supplemented with 10% FBS were plated at 4 x 10^5 cells/well in 6-well dishes for 48 hours to allow complete attachment of cells to the substratum. Culture medium was then changed to low (0.5 mM; open symbols) or normal (1.5 mM; filled symbols) Ca^{2+} Ham's F12 supplemented with 5% FBS and aliquots taken and assayed for PHF by ELISA at the indicated time points. Time courses for PHF secretion in different EC Ca^{2+} conditions are shown for both SHR (a) and WKY (b) parathyroid cells. Data points represent mean PHF ± SEM of four separate experiments each with 6 culture dishes/treatment group. *Value is significantly different ($P<0.05$; t-test) from 1.5 mM Ca^{2+} data point at the indicated time point.

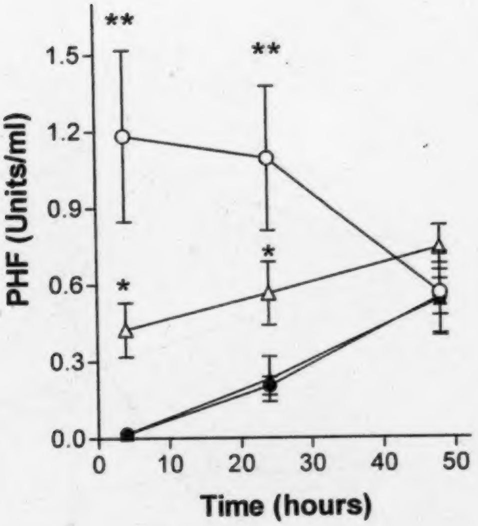

Figure 4-3—Time course for effect of extracellular Ca^{2+} on PHF release in parathyroid organ culture. Parathyroids were harvested from SHR and WKY rats and placed in 24-well culture plates (two glands from one rat/well) containing either low (0.5 mM; open symbols) or normal (1.5 mM; filled symbols) Ca^{2+} Ham's F12 supplemented with 5% FBS. Aliquots were taken and assayed for PHF by ELISA at the indicated time points. Time courses for PHF secretion under different EC Ca^{2+} conditions are shown for both SHR (circular symbols) and WKY (triangle symbols) parathyroid cells. Data points represent mean PHF ± SEM of 4 culture wells representing 4 animals for each of the experimental conditions. * Indicates significantly different vs corresponding 1.5 mM EC Ca^{2+} at the indicated time point ($P<0.05$; one-way ANOVA); ** indicates significantly different vs 0.5 mM EC Ca^{2+} WKY PTG group ($P<0.05$; one-way ANOVA).

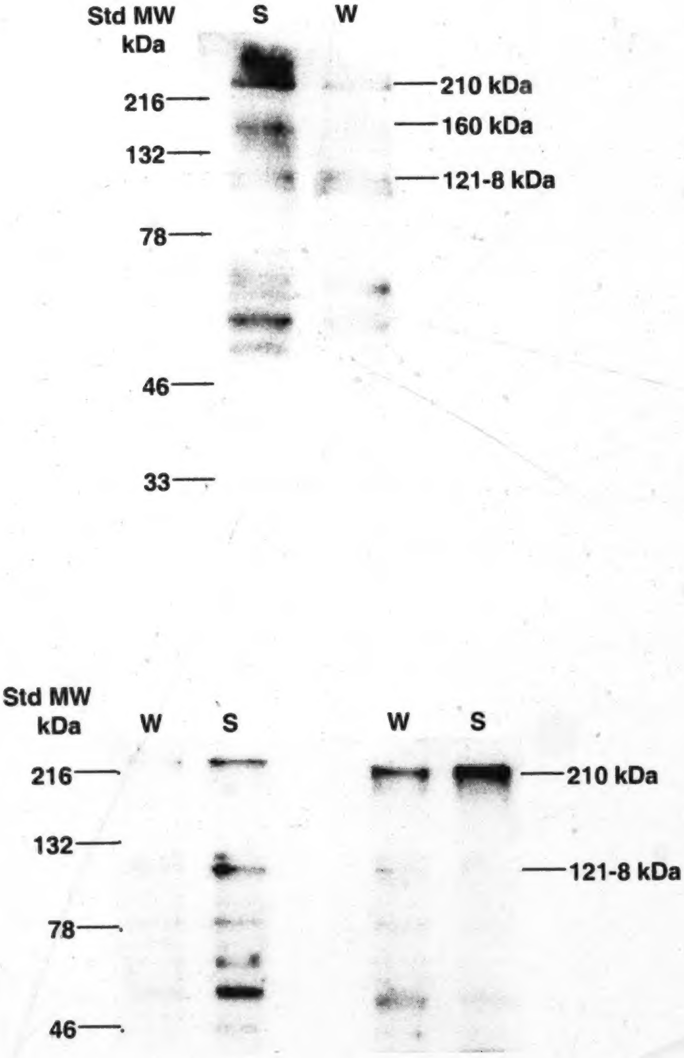
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Figure 4-4—CaR protein in SHR and WKY parathyroid cells. Western blots for CaR were performed on 2.5 μg whole parathyroid cell extracts for both SHR (S) and WKY (W) parathyroid cells at the same cell density (representing approximately 60-70% confluency) and compared within the same blots. Results shown are of 3 separate experiments.

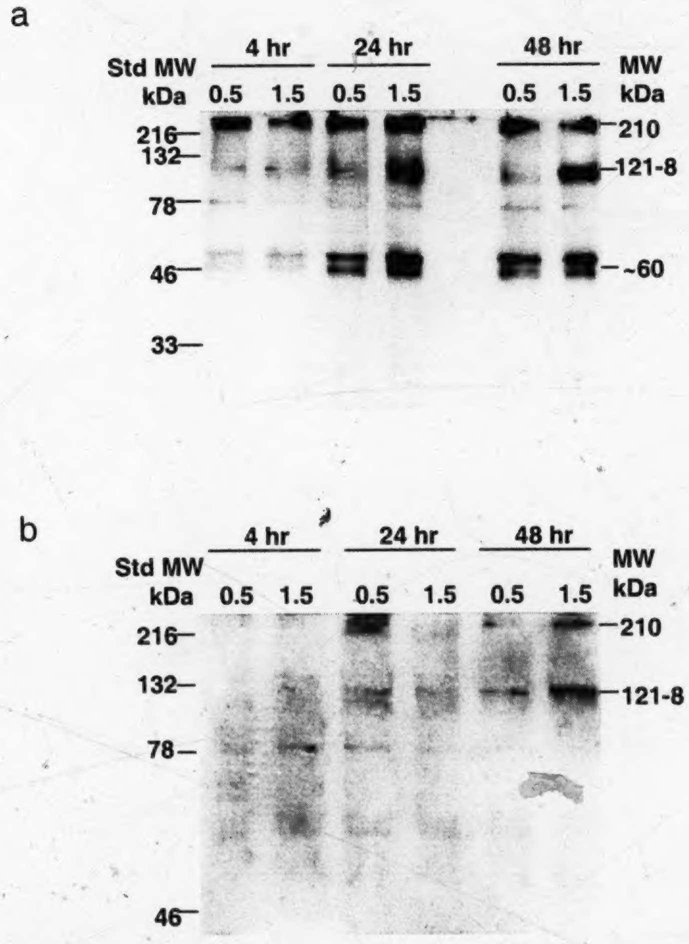
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Figure 4-5—Time course for and effect of extracellular Ca^{2+} on CaR expression in parathyroid cells. SHR (a) and WKY (b) parathyroid cells were seeded at a low density and allowed to attach for 48 hours. Medium was then replaced with either low (0.5) or normal (1.5) mM Ca^{2+} Ham's F12 supplemented with 5% FBS and cell lysate collected at the indicated time points (4, 24, 48 hours) for CaR determination by Western blotting. Result shown is a representative blot of 3 identical experiments yielding similar results.

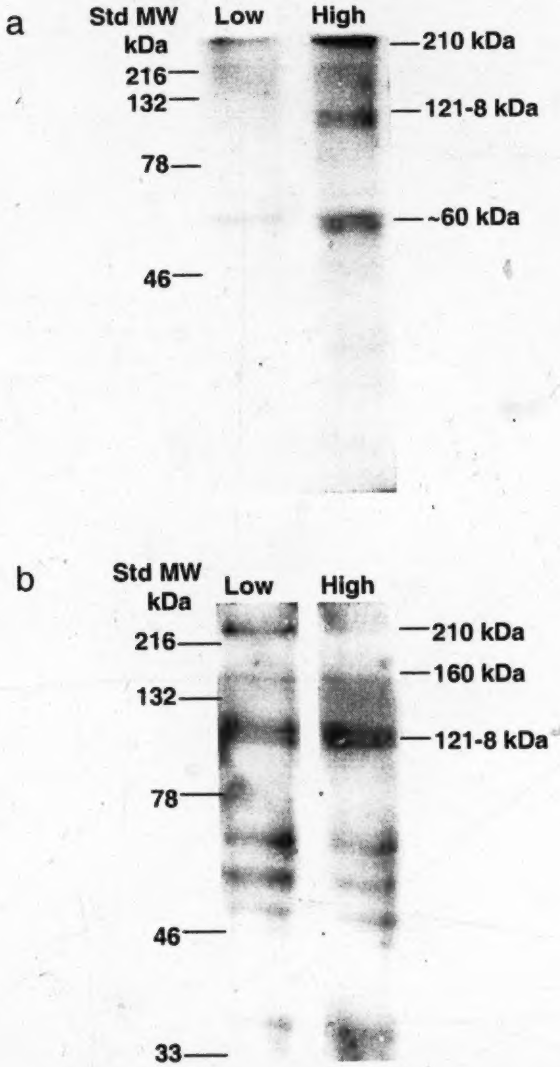
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Figure 4-6—Effect of parathyroid cell culture density on CaR protein expression. Parathyroid cells derived from both SHR (S) and WKY (W) were seeded at either a low (5×10^4 cells/55 x 10 cm culture dish) or high (2.5×10^5 cells/55 x 10 cm culture dish) density and allowed to attach for 48 hours in DMEM containing 5% FBS. Cell lysates were then collected after a further 24 hours of growth in 1.5 mM Ca^{2+} Ham's F12 and analyzed for CaR protein by Western blotting.

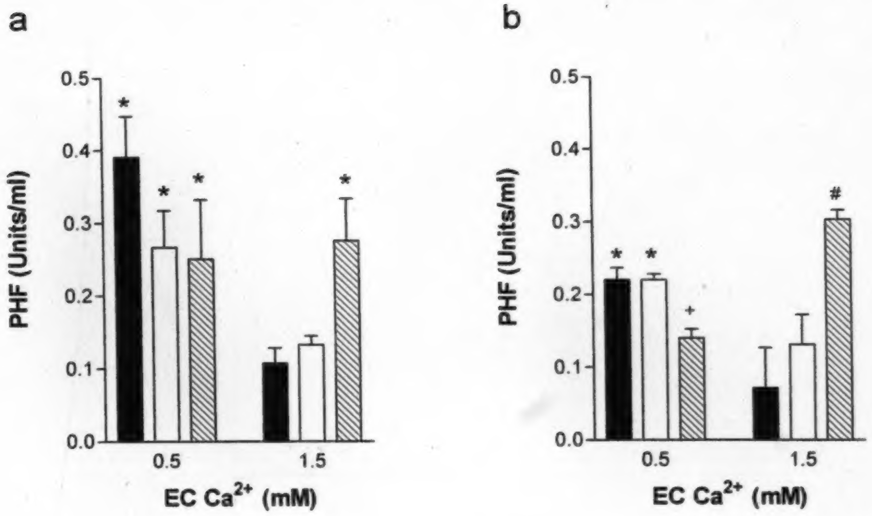

Figure 4-7—Role of the CaR in effects of extracellular Ca^{2+} on PHF release. Parathyroid cells derived from SHR (a) and WKY (b) rats were exposed to PBS containing BSA (1.4 μg/ml) (solid bars) or primary anti-CaR antibody alone (1.4 μg/ml) (hatched bars) or previously pre-absorbed (with control CaR peptide) anti-CaR antibody (unshaded bars) for 60 minutes. Cells were then washed and media replaced with either low (0.5) or normal (1.5) mM Ca^{2+} Ham's F12 supplemented with 5% FBS for 60 minutes and samples collected for PHF analysis in ELISA. Results shown indicate the mean PHF ± SEM for 3 separate experiments. * Indicates significantly different from control 1.5 mM Ca^{2+} group ($P < 0.05$; t-test); # indicates significantly increased vs control 0.5 mM Ca^{2+} group ($P < 0.05$; t-test); + indicates significantly decreased vs control 0.5 mM Ca^{2+} group with ($P < 0.05$; t-test).

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Chapter 5

Regulation of parathyroid hypertensive factor secretion by vitamin D3 analogs in parathyroid cells derived from spontaneously hypertensive rats[1]

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5 EFFECT OF VITAMIN D3 ANALOGS ON PHF SECRETION

5.1 Introduction

The hormonally active form of vitamin D3, 1,25-$(OH)_2D_3$, in concert with PTH, plays a critical role in plasma Ca^{2+} and phosphate homeostasis. Hypocalcemia, directly and indirectly via stimulation of PTH release, stimulates accumulation of 1,25-$(OH)_2D_3$ through activation of kidney 25-hydroxyvitamin D3-1α-hydroxylase (1αOHase) and hydroxylation of the circulating precursor 25-hydroxyvitamin D3 (1-3). 1,25-$(OH)_2D_3$ functions to increase the pool of available plasma Ca^{2+} by stimulating absorption in the intestine, reabsorption in the kidney, and under low dietary calcium intake conditions, bone remodeling and resorption (1-3). Part of the negative feedback control of this endocrine system occurs in the PTG, where 1,25-$(OH)_2D_3$ transcriptionally (4) suppresses PTH synthesis, inhibits PTH release, and inhibits parathyroid cell proliferation. In addition to the classical roles of 1,25-$(OH)_2D_3$, there are also many non-calcemic functions. Some of these include antiproliferative and pro-differentiative effects in promyelocyte HL-60 cells (5), keratinocytes (6), and vascular smooth muscle cells (7).

An extensive non-classical role for 1,25-$(OH)_2D_3$ has also been defined in the cardiovascular system. Amongst the cardiovascular effects of 1,25-$(OH)_2D_3$ are positive inotropism in cardiomyocytes (8) and stimulation of L-type Ca^{2+} channel current and increased intracellular Ca^{2+} (9) in vascular smooth muscle cells. Abnormalities in both the cardiovascular function and the plasma levels of 1,25-$(OH)_2D_3$ have implicated a role for 1,25-$(OH)_2D_3$ in experimental and human hypertension. Plasma 1,25-$(OH)_2D_3$ has been shown to be elevated in low-renin and salt-sensitive human hypertension (10) as well as in several rat models of low-renin hypertension (11-13). Furthermore, several studies have shown that chronic 1,25-$(OH)_2D_3$ administration potentiates induced contractility *in vitro* in vascular tissue (14) and in mesenteric arteries taken from the SHR (15), but not from the genetic control strain, WKY rats. Not surprisingly then, a role for 1,25-$(OH)_2D_3$ in blood pressure regulation in hypertension has been suggested (16) and transient effects on systolic blood pressure in male hypertensive patients (but not in normotensives) have been observed (17). However chronic treatment with 1,25-$(OH)_2D_3$ was not shown to exert any significant effect on blood pressure in the SHR (14).

Since 1,25-$(OH)_2D_3$ does not appear to have chronic effects on blood pressure, it has been suggested that 1,25-$(OH)_2D_3$ may modulate other endocrine systems which produce more long-term hypertensive effects. Parathyroid Hypertensive Factor (PHF), a substance of parathyroid origin which may contribute to hypertension by potentiating the intracellular calcium-raising effects of classical vasoconstrictors, was thought to be a potential candidate for regulation by 1,25-$(OH)_2D_3$ (for review see (18)). PHF is elevated in the same subset of hypertensives, i.e. low-renin, salt-sensitive, that 1,25-$(OH)_2D_3$ is found to be elevated (for review see (18)). As the PTG is a known target for 1,25-$(OH)_2D_3$, it was hypothesized that 1,25-$(OH)_2D_3$ would stimulate secretion of PHF.

We report here that 1,25-$(OH)_2D_3$ indeed stimulates PHF release rapidly and sub-acutely with enhanced sensitivity in parathyroid cells derived from SHR relative to the normotensive control strain. Conversely, the metabolite 24,25-$(OH)_2D_3$ was shown to rapidly and sub-acutely inhibit PHF release with blunted sensitivity in cells derived from SHR. Furthermore, by utilizing the $1,25VDR_{nuc}$–specific agonist analog BT (1,24$(OH)_2$-

22-ene-24-cyclopropyl-D_3) which is incapable of inducing the rapid effects of 1,25-$(OH)_2D_3$ observed in some systems (19), we will provide evidence suggesting the rapid effect of 1,25-$(OH)_2D_3$ on PHF release may be mediated by the newly described 1,25VDR_{mem}.

5.2 Materials & Methods

5.2.1 Materials

Mouse anti-PHF oligoclonal antibody (IgM-3A), standard semi-pure PHF, and PHF-horseradish peroxidase conjugate (PHF-HRP) were prepared as described previously (20). Monoclonal anti-1,25VDR_{nuc} IgG (clone 9A7) was purchased from Affinity Bioreagents Inc. (Golden, CO, USA) and monoclonal anti-Histone H1 IgG (clone AE-4) was purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Anti-1,25VDR_{mem} antisera Ab99 was generated as described previously (21, 22). Vitamin D3 analogs 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 were obtained from Roussell UCLAS and Sigma Aldrich Chemical Company (St. Louis, MO, USA) and 1,24$(OH)_2$-22-ene-24-cyclopropyl-D_3 (BT) was a generous gift from Leo Pharmaceuticals (Copenhagen, Denmark). All cell culture media were obtained from Gibco/BRL (Burlington, ON). BCA protein assay reagents were obtained from Pierce Chemical Company (Rockford, IL, USA) and acrylamide bis, temed, ammonium persulfate, and kaleidoscope molecular weight standards were all purchased from BioRad (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membranes (Immobilon-P) were obtained from Millipore (Bedford, MA, USA) and enhanced chemiluminescence

(ECL) detection reagents for Western blot analysis were obtained from Amersham (Arlington Heights, IL, USA). WST-1 cell proliferation reagent was obtained from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals were purchased from either Sigma/Aldrich (St. Louis, MO, USA) or Fisher chemicals (Fairlawn, NJ, USA). SHR and WKY rats were obtained from Charles River (St. Constant, Quebec, Canada).

5.2.2 PHF Secretion in Response to Vitamin D3 Analogs

For investigation of the effects of different vitamin D3 analogs on PHF secretion, both parathyroid cell and organ culture models were employed. Parathyroid cell suspensions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS were obtained as previously described (23), seeded in 96-well plates for approximately 48 hours to achieve a final cell density of 1.0 x 10^4 cells/well. Fresh solutions of vitamin D3 analogs were prepared in ethanol, followed by dilution in culture medium (Ham's F12 adjusted to 1.5 mM Ca^{2+} and supplemented with 5% FBS) so that the final concentration of ethanol was less than 0.05% (v/v). Media were then gently aspirated and replaced with media containing vitamin D3 analog or vehicle alone and incubated for either 30 minutes or 48 hours at 37 C. Aliquots of 20-50 μl/well were then collected and processed for PHF analysis as previously described (24), media aspirated and replaced with phenol red-free fresh culture medium (100 μl/well) and analyzed for cell viability as described below. For experiments involving parathyroid organ culture, glands were isolated from SHR and WKY rats as described previously (23) and placed in 24-well culture dishes (2 glands/well) containing 250 μl/well culture medium containing

$1,25\text{-}(OH)_2D_3$ prepared as described above. Aliquots of 10 μl were removed at various time points for PHF detection (24). Use of animals was according to CCAC guidelines, and reviewed and approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

5.2.3 Sub-cellular Fractionation and Western Blots for $1,25VDR_{mem}$ and $1,25VDR_{nuc}$ protein

Parathyroid cells cultured in 150 x 10 mm tissue culture dishes were washed twice with Hank's balanced salt solution (HBSS) and 400 μl of a lysis buffer (15 mM Tris, 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, pH 7.4) was added. For whole cell extracts, the lysis buffer also contained 0.5 % NP-40 and the cell lysate was harvested and frozen at -70 C. To obtain sub-cellular fractions, cell lysate was homogenized and sonicated for 5 minutes, then nuclei and cell debris were pelleted by centrifugation at 750 x g for 5 minutes and reconstituted with lysis buffer containing 0.5% NP-40 after the supernatant was collected. The supernatant was ultracentrifuged for 30 minutes at 100 000 x g and 4 C and the resulting supernatant representing the cytosolic fraction was decanted and the plasma membrane pellet washed two times with PBS and solubilized with lysis buffer containing 0.5% NP-40. Aliquots of all three sub-cellular fractions were frozen at -70 C until Western blot analysis. Protein content of the samples was measured using the BCA protein assay (Pierce) and samples were diluted in reducing treatment sample buffer and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as previously described by Laemmli (25). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting at

75 V for 1.5 hours in transfer buffer (19 mM Tris-HCl, 150 mM glycine, 20% methanol) and immunodetection of specific bands carried out according to the Western blotting protocol supplied by Millipore with the PVDF membranes. Primary antibody concentrations were 1:1000 for rabbit anti-1,25VDR$_{mem}$ IgG (Ab99), 1:1000 for rat anti-1,25VDR$_{nuc}$ IgG (9A7), or 1:500 mouse anti-histone H1 (AE-4). All appropriate secondary antibodies were used at a dilution of 1:2000 and specific proteins were visualized using the enhanced chemiluminescence (ECL) detection system.

5.2.4 Statistical Analysis

ELISA standard curves and unknown values were determined using the Microplate Manager/PC software (BioRad Laboratories). Results are expressed as the mean ± SEM and statistical evaluation for differences between treatment groups was performed using either one-way ANOVA (Student-Newman-Keuls test) unless indicated otherwise. $P < 0.05$ was taken to be significant.

5.3 Results

5.3.1 Effect of 1,25-$(OH)_2D_3$ on PHF Secretion

The rapid and sub-acute effects of 1,25-$(OH)_2D_3$ on PHF release from cultured parathyroid cells are shown in Figure 5-1. Exposure to 1,25-$(OH)_2D_3$ for 30 minutes resulted in a dose-dependent stimulation of PHF release from parathyroid cells derived from both SHR (Fig. 5-1a) and WKY (Fig. 5-1b) rats. The dose-response curves followed a sigmoidal pattern and the SHR parathyroid cells displayed increased sensitivity to 1,25-

$(OH)_2D_3$ as indicated by the lower EC50 (1.52×10^{-8} M versus 9.64×10^{-8} M for WKY) and the leftward shift of the dose-response curve. Extension of the incubation time to 48 hours resulted in maintained dose-dependent stimulation of PHF secretion by 1,25-$(OH)_2D_3$, although there was no difference in the sensitivity between strains as both SHR (Fig. 5-1c) and WKY (Fig. 5-1d) dose response curves yielded similar EC50s (SHR PTC: 1.14×10^{-7} M versus WKY PTC: 1.04×10^{-7} M for WKY).

Figure 5-2 shows the effect of 1,25-$(OH)_2D_3$ on PHF release from intact PTG organ culture. 1,25-$(OH)_2D_3$ stimulated PHF secretion from SHR and WKY PTG in a similar manner as for cultured parathyroid cells. PTG were rapidly stimulated by 1 μM 1,25-$(OH)_2D_3$ to secrete PHF after 4 hours exposure for both SHR and WKY PTG cultures (Fig. 5-2a). However after 48 hours, although mean PHF was increased, 1 μM 1,25-$(OH)_2D_3$ did not significantly stimulate PHF release for SHR and WKY PTG cultures (Fig. 5-2b).

Taken together, these results indicate that 1,25-$(OH)_2D_3$ rapidly and sub-acutely stimulates PHF release from parathyroid cells and that sensitivity is enhanced in parathyroid cells derived from SHR animals.

5.3.2 Effect of 24,25-$(OH)_2D_3$ on PHF Secretion

To determine whether effects of 1,25-$(OH)_2D_3$ on PHF release were vitamin D3 analog specific, the effect of 24,25-$(OH)_2D_3$ on basal PHF secretion was examined. In contrast to 1,25-$(OH)_2D_3$, exposure to 24,25-$(OH)_2D_3$ for 30 minutes resulted in a dose-dependent inhibition of basal PHF release from parathyroid cells derived from both SHR (Fig. 5-3a) and WKY (Fig. 5-3b) rats. The SHR PTC dose-response curve followed a sigmoidal pattern with an IC50 of 5.06 x 10^{-9} M, however the WKY PTC dose-response curve revealed an unusual logarithmic relationship between 24,25-$(OH)_2D_3$ concentration and PHF secretion which indicated increased sensitivity to 24,25-$(OH)_2D_3$ relative to the SHR PTC. Extension of the incubation time to 48 hours resulted in maintained dose-dependent inhibition of PHF secretion by 24,25-$(OH)_2D_3$, although at this point, the dose-response curve for SHR PTC (Fig. 5-3c) displayed the unusual logarithmic pattern and the WKY PTC (Fig. 5-3d) dose response curve followed the expected sigmoidal pattern and yielded an IC50 of 4.70 x 10^{-10} M.

Taken together, these results indicate that 24,25-$(OH)_2D_3$ rapidly and sub-acutely inhibits basal PHF release from parathyroid cells and that sensitivity may be decreased in parathyroid cells derived from SHR animals.

5.3.3 VDR protein expression

Because parathyroid cells derived from either SHR or WKY rats displayed differential regulation of PHF release by 1,25-$(OH)_2D_3$, we examined the parathyroid expression of the classical $1,25VDR_{nuc}$ as well as the putative $1,25VDR_{mem}$. Figure 5-4a shows $1,25VDR_{mem}$ protein expression, indicated by a single band at approximately 66 kDa, is enhanced in sub-cultured SHR parathyroid cells versus cells derived from the normotensive strain, WKY. However, WKY parathyroid cells were shown to have slightly increased or equivalent levels of $1,25VDR_{nuc}$ protein versus SHR parathyroid cells (Figure 5-4b). Further characterization of the $1,25VDR_{mem}$ in SHR and WKY PTC according to sub-cellular localization is shown in Figure 5-5a. The $1,25VDR_{mem}$ seemed to represent a similar proportion of total protein in nuclear, cytosolic, and plasma membrane fractions prepared from whole parathyroid cell lysates for both SHR and WKY PTC except for a reduction in band intensity for the plasma membrane fraction of WKY PTC. Figure 5-5b shows that the sub-cellular distribution of the nuclear marker, Histone H1, is nuclear and cytosolic indicating the plasma membrane fraction is largely free of nuclear protein contaminants.

Earlier studies have reported that the Ab99 does not cross-react with recombinant $1,25VDR_{nuc}$ (26). We have confirmed this observation by running duplicate samples and purified $1,25VDR_{nuc}$ on 8% SDS-PAGE gels and Western blotting half of the blot with anti-$1,25VDR_{nuc}$ (9A7) and the other to anti-$1,25VDR_{mem}$ (Ab99). The result is shown in Figure 5-6. Antibody 9A7 was shown to recognize two bands, one at approximately 66 kDa and another at the expected MW of 53 kDa. Probing with the anti-$1,25VDR_{mem}$ antisera resulted in only a 66 kDa visible band. Excess pure recombinant $1,25VDR_{nuc}$ was not recognized by Ab99. These data suggest that the $1,25VDR_{mem}$ and $1,25VDR_{nuc}$ are of different molecular weights and that Ab99 recognizes an epitope of the $1,25VDR_{mem}$ which is either not present or is masked in the $1,25VDR_{nuc}$.

5.3.4 Role of the VDRs in mediating the effects of $1,25\text{-}(OH)_2D_3$ on PHF Secretion

To examine the extent of involvement of the $1,25VDR_{mem}$ and classical $1,25VDR_{nuc}$ in mediating the effects of $1,25\text{-}(OH)_2D_3$ on PHF release, the vitamin D3 analog $1,24(OH)_2$-22-ene-24-cyclopropyl-D_3 (BT) was used. This vitamin D3 analog has high affinity for the $1,25VDR_{nuc}$, but was shown to be ineffective in stimulating a variety of rapid, non-genomic effects in other systems (19). Exposure of SHR and WKY PTC to

100 nM BT had no effect on PHF release after either 30 minutes (Fig. 5-7a) or 48 hours (Figure 5-7b) in culture. These results strongly suggest that the $1,25VDR_{nuc}$ is not involved in mediating the rapid and sub-acute effects of $1,25\text{-}(OH)_2D_3$ on PHF secretion from SHR or WKY parathyroid cells.

5.4 Discussion

In this study, the role of different vitamin D3 analogs in regulation of secretion of PHF from both physiological and pathological parathyroid tissue was examined. $1,25\text{-}(OH)_2D_3$ was considered an obvious candidate hypothesized to directly stimulate PHF secretion based on the plasma profile of elevated $1,25\text{-}(OH)_2D_3$ and PHF in low-renin hypertension, and the classical role of $1,25\text{-}(OH)_2D_3$ in regulation of the PTG. In addition, parathyroid cells derived from the hypertensive model, SHR, were predicted to display enhanced sensitivity to $1,25\text{-}(OH)_2D_3$. This study characterized the rapid and sub-acute stimulatory effects of $1,25\text{-}(OH)_2D_3$ and inhibitory effects of $24,25\text{-}(OH)_2D_3$ on PHF release and revealed that SHR parathyroid cells displayed enhanced sensitivity to $1,25\text{-}(OH)_2D_3$ and reduced sensitivity to $24,25\text{-}(OH)_2D_3$. This is also the first demonstration of the parathyroid expression of the newly described $1,25VDR_{mem}$. Furthermore, PHF secretion studies using the classical $1,25VDR_{nuc}$ agonist BT revealed that the $1,25VDR_{nuc}$ was not likely to be mediating the stimulatory effects of $1,25\text{-}(OH)_2D_3$ on PHF release, leaving open the possibility of the $1,25VDR_{mem}$ mediating these rapid effects in the parathyroid. These data support the idea that vitamin D3 metabolites and altered sensitivity to them with respect to PHF secretion contributes to the elevated

PHF status of the SHR.

The original focus of this work was to examine and characterize the potential role of the biologically active vitamin D3 metabolite, 1,25-$(OH)_2D_3$, in regulation of PHF release. Both parathyroid cultured cells and intact glands were more responsive to rapid regulation, as opposed to sub-acute regulation, of PHF secretion by 1,25-$(OH)_2D_3$. In SHR parathyroid cell cultures, enhanced sensitivity to 1,25-$(OH)_2D_3$ was observed at 30 minutes versus the 48 hour time point suggesting that the primary effect of 1,25-$(OH)_2D_3$ on PHF release occurs rapidly and that the lingering effects of this rapid release are maintained by 48 hours. Conversely, sensitivity to inhibition of PHF release by 24,25-$(OH)_2D_3$ was unchanged in 30 minutes versus 48 hours for SHR parathyroid cells; however, WKY parathyroid cells displayed increased sensitivity after 30 minutes versus 48 hours exposure to 24,25-$(OH)_2D_3$. Similar results were observed for parathyroid organ cultures with significant effects of 1,25-$(OH)_2D_3$ after 4, but not 48 hours. Other regulators such as EC Ca^{2+} (24) have also been shown to exert only rapid effects on PHF release which suggests that PHF may be stored, and that regulation of release largely occurs post-transcriptionally through modulation of the secretory pathway rather than by altering PHF synthesis.

Rapid effects of 1,25-$(OH)_2D_3$ (and other steroids) have been described in many systems and cancerous cell lines and is reviewed elsewhere (27). In parathyroid cells, rapid responses to 1,25-$(OH)_2D_3$ such as increased cytosolic Ca^{2+} (28) and phospholipid metabolism (29) have been described. It is possible that these effects may be mediated by the classical nuclear vitamin D3 receptor, which is expressed in PTG (30, 31) and maintained in sub-cultured parathyroid cells (32). However, it is more likely that the putative $1,25VDR_{mem}$, which has been shown to mediate other rapid effects of 1,25-$(OH)_2D_3$ such as stimulation of PKC and antiproliferative effects in growth zone chondrocytes (21, 33), is involved. The effects of 1,25-$(OH)_2D_3$ on parathyroid cytosolic Ca^{2+} were insensitive to either diltiazem or verapamil which led the authors to conclude that 1,25-$(OH)_2D_3$ may be affecting Ca^{2+} entry through direct interaction with the cell plasma membrane and not through modulation of voltage-sensitive Ca^{2+} channels (28). This suggests that a receptor-operated Ca^{2+} channel, possibly involving the $1,25VDR_{mem}$, may be involved in mediating the rapid 1,25-$(OH)_2D_3$–induced Ca^{2+} entry.

To eliminate the possibility that the $1,25VDR_{nuc}$ is involved in regulation of PHF secretion, we employed the vitamin D3 analog BT, a classical $1,25VDR_{nuc}$ agonist which is incapable of exerting the rapid effects of 1,25-$(OH)_2D_3$ observed in some systems (19). It is not known if BT can bind the $1,25VDR_{mem}$ or act as an agonist for this receptor, however the lack of effect of BT on PHF release suggests a $1,25VDR_{nuc}$-independent mechanism of action for 1,25-$(OH)_2D_3$ regulation of PHF secretion. This leaves open the possibility of the $1,25VDR_{mem}$ for transduction of these rapid effects.

Parathyroid cells derived from SHR appeared to display increased sensitivity to stimulation of PHF secretion by 1,25-$(OH)_2D_3$ after 30 minutes versus cells derived from the normotensive WKY strain. There are several possible explanations for either differences in maximal response or sensitivity to 1,25-$(OH)_2D_3$ between the strains with respect to PHF release. Enhanced sensitivity in SHR parathyroid cell cultures may indicate altered post-vitamin D3 receptor signal transduction pathways. Western blot analysis showed increased $1,25VDR_{mem}$ in SHR parathyroid cells compared to WKY, but no differences or even slightly decreased expression of the $1,25VDR_{nuc}$ protein. This may account for the increased PHF secretory response to 1,25-$(OH)_2D_3$ in SHR parathyroid cells if the 1,25VDR$_{mem}$ in fact mediates the stimulatory effects of 1,25-$(OH)_2D_3$ on PHF release. If this expression pattern is also observed *in vivo* this could at least partly account for the elevated plasma PHF observed in SHR. Earlier 1,25-$(OH)_2D_3$ binding studies showed increased maximal specific binding capacity for calcitriol in SHR parathyroid tissue suggesting increased receptor expression (34). Since we have shown that expression of the 1,25VDR$_{nuc}$ is unchanged between strains or slightly reduced for SHR parathyroid cells, it is likely that the enhanced binding they observed was due to the 1,25VDR$_{mem}$. A second explanation for enhanced sensitivity to 1,25-$(OH)_2D_3$ in the SHR parathyroid may be due to a prolonged half-life due to either alterations in 1,25-$(OH)_2D_3$ release from vitamin D3 binding proteins or decreased activity of cytochrome P450 hydroxylases which are known to metabolically inactivate free 1,25-$(OH)_2D_3$ in parathyroid cells (32). A final possibility is that alterations in the calcitriol receptor involved in mediating these effects or the downstream signaling pathways, if the effects are indeed receptor-mediated, may be responsible for enhanced sensitivity of SHR parathyroid tissue to 1,25-$(OH)_2D_3$.

The observation that the 1,25VDR$_{mem}$, but not the 1,25VDR$_{nuc}$ was differentially expressed in the SHR versus WKY parathyroid led us to investigate some of the characteristics of the 1,25VDR$_{mem}$ protein. Western blotting analysis indicated that the 1,25VDR$_{mem}$ is slighter larger than the 1,25VDR$_{nuc}$ (66 kDa versus 53 kDa). Furthermore, as previously reported (26, 33), anti-1,25VDR$_{mem}$ antisera does not cross-react with excess pure 1,25VDR$_{nuc}$ indicating the antibody recognizes an epitope not present or possibly not exposed in the native 1,25VDR$_{nuc}$. This may mean that the

$1,25VDR_{mem}$ is a novel protein unrelated to the $1,25VDR_{nuc}$ and arises from a distinct gene, or that it is a modified $1,25VDR_{nuc}$ or alternative splicing product from the $1,25VDR_{nuc}$ gene which contains a sequence not found or not conformationally exposed in the $1,25VDR_{nuc}$. However, the anti-$1,25VDR_{nuc}$ antibody recognizes a protein of approximately the same molecular weight as the $1,25VDR_{mem}$ in parathyroid extracts suggesting that the $1,25VDR_{mem}$ and $1,25VDR_{nuc}$ may be related. The parathyroid sub-cellular distribution of the $1,25VDR_{mem}$ was also briefly investigated. In chick enterocytes, Nemere et al found predominant localization of the $1,25VDR_{mem}$ in basolateral membranes, followed by endoplasmic reticulum (26), however, we observed roughly equal proportions of the $1,25VDR_{mem}$/total protein in crude nuclear, cytosolic, and cell plasma membrane preparations. This may indicate different stages of receptor processing or may be related to our method of purification. This method allowed for any unlysed cells and cell debris to be pelleted and included with the nuclear fraction and cytosolic fractions were also found to contain a significant amount of nuclear-derived protein. However, the cell plasma membrane fraction was found to be free of nuclear-derived protein (histone H1). Therefore $1,25VDR_{mem}$ detected in this fraction likely originated in the cell plasma membrane.

In this study, we also found that 24,25-$(OH)_2D_3$ has rapid effects on PHF secretion from both SHR and WKY parathyroid cells. Unlike 1,25-$(OH)_2D_3$, the effects of 24,25-$(OH)_2D_3$ on PHF release were inhibitory. Opposing effects of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ have often been described for several tissues where these steroids have been found to exert rapid effects (35, 36). It is not known whether the effects of 24,25-$(OH)_2D_3$ on PHF release observed here are receptor-mediated, but a novel membrane receptor for 24,25-$(OH)_2D_3$ (24,25VDR_{mem}) distinct from the 1,25VDR_{mem} (37), has been identified and characterized in chondrocytes (33, 37), osteoblasts (37), and enterocytes (38). To our knowledge, a specific antibody to this receptor is not yet available but it will be interesting to examine expression of this putative receptor in the parathyroid of both SHR and WKY rats. It is also possible that 24,25-$(OH)_2D_3$ exerts its effects via a calcitriol receptor or that 24,25-$(OH)_2D_3$ acts through a non-receptor-mediated pathway perhaps by altering membrane composition or signaling via lipid rafts.

The dose-response curves for regulation of PHF secretion by 24,25-$(OH)_2D_3$ exhibited some irregular features. After 30 minutes exposure, the dose-response curve for WKY, but not SHR, parathyroid cells displayed an unusual non-sigmoidal logarithmic pattern, whereas after 48 hours, SHR, but not WKY parathyroid cells responded in this unusual manner. This may indicate the involvement of multiple receptors such as those mentioned above. These receptors may be differentially expressed between strains and/or may have temporal component to their expression patterns. It is possible that high concentrations of 24,25-$(OH)_2D_3$ can activate the 1,25VDR_{mem} resulting in stimulation of PHF release. Combined inhibitory and stimulatory effects in response to high concentrations of 24,25-$(OH)_2D_3$ could account for the absence of a plateau in this concentration range. Alternatively, 24,25-$(OH)_2D_3$ may modulate autocrine/paracrine regulators of PHF release resulting in a non-classical dose-response curve. A third, but less likely, possibility is that 24,25-$(OH)_2D_3$ is converted to 1,24,25-$(OH)_3D_3$ by parathyroid cytochrome P450 hydroxylases and this metabolite can modulate PHF secretion.

The regulation of PHF release by vitamin D3 metabolites and the abnormalities in parathyroid sensitivity to these metabolites in the SHR may play a significant role in the etiology of hypertension in this strain, and the findings may possibly extend to low-renin hypertension. When hypertensive patients are classified according to plasma renin activity, the low-renin subset (representing approximately 30-40% of essential hypertension) exhibits, concurrent with elevated PHF, a plasma calcium deficit accompanied by appropriate shifting of the calcium-regulating hormones resulting in increased plasma PTH, elevated 1,25-$(OH)_2D_3$, and suppressed calcitonin (39). In the Dahl-salt sensitive rat (12), elevated 1,25-$(OH)_2D_3$ precedes the onset of hypertension. In the SHR, a model of hypertension which has been reported to exhibit low plasma renin activity (40), the 1,25-$(OH)_2D_3$ status appears to depend on a variety of factors (41). Nonetheless, there have been several reports of elevated 1,25-$(OH)_2D_3$ coincident with (42) or preceding the development of hypertension (42, 43); although there appears to be an age-related decline in 1,25-$(OH)_2D_3$ (44). Taken together, these findings suggest that vitamin D3 metabolites and altered sensitivity to them in the PTG may play a key role in the development of hypertension in the SHR through abnormal regulation of PHF release.

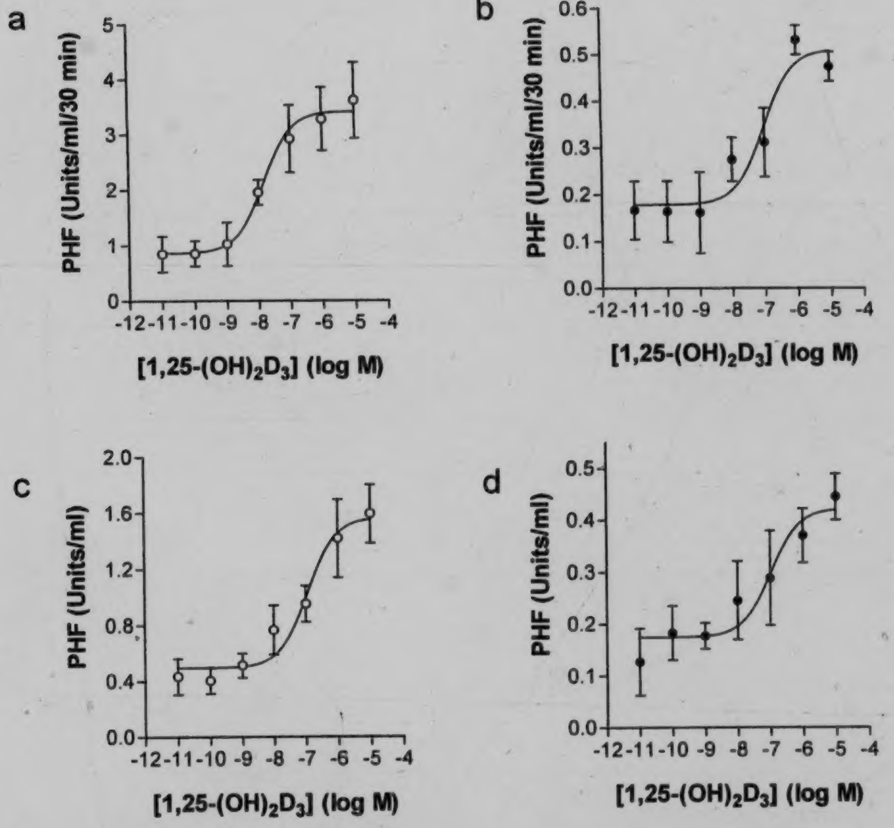

Figure 5-1—Effect of 1,25-$(OH)_2D_3$ on PHF secretion from sub-cultured parathyroid cells. For investigation of the effect of 1,25-$(OH)_2D_3$ on cumulative PHF release after 30 minutes (a, b) and 48 hours (c,d), parathyroid cell suspensions in DMEM supplemented with 5% FBS were seeded in 96-well plates at a density of approximately 1×10^4 cells/well. After a 48 hour attachment period, media were replaced with 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS containing the indicated concentrations of 1,25-$(OH)_2D_3$ or ethanol vehicle (control) and incubated for 30 minutes or 48 hours at 37 °C. Aliquots were then collected and processed for PHF analysis, media aspirated and replaced with fresh 1.5 mM Ca^{2+} Ham's F12 medium + 5% FBS and analyzed for cell viability. Results shown are for SHR (a, c) and WKY (b, d) parathyroid cells and are presented as the mean ± SEM of 3 experiments with at least 6 replicate wells per treatment group for each experiment.

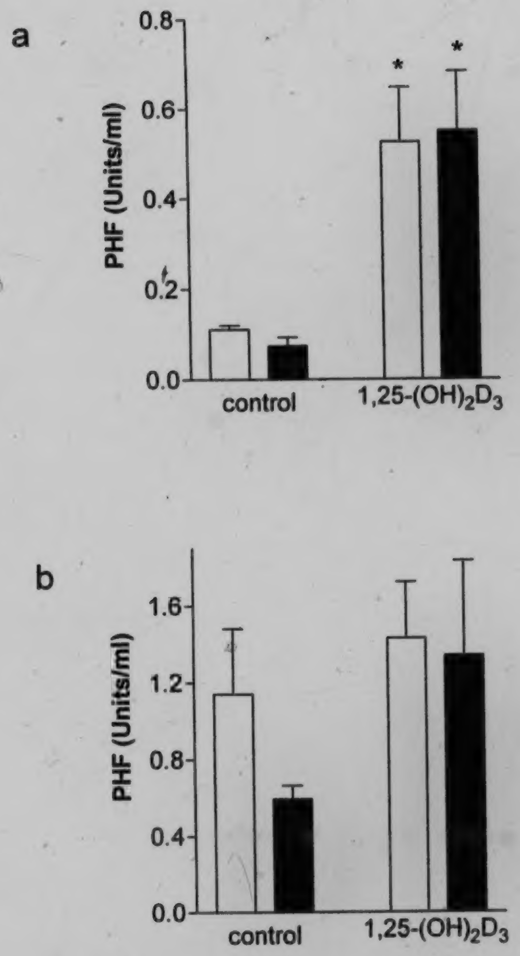

Figure 5-2—Effect of 1,25-$(OH)_2D_3$ on PHF release in parathyroid organ culture. Parathyroids were harvested from SHR (unshaded bars) and WKY (shaded bars) rats and placed in 24-well culture plates (two glands/well) containing 1 μM 1,25-$(OH)_2D_3$ or ethanol vehicle (control) in 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS. Aliquots were taken after 4 hours (a) and 48 hours (b) and assayed for PHF by ELISA. Data points represent mean PHF ± SEM of 4 culture wells representing 4 animals for each of the experimental conditions. *Indicates significantly different vs control group ($P < 0.05$; t-test).

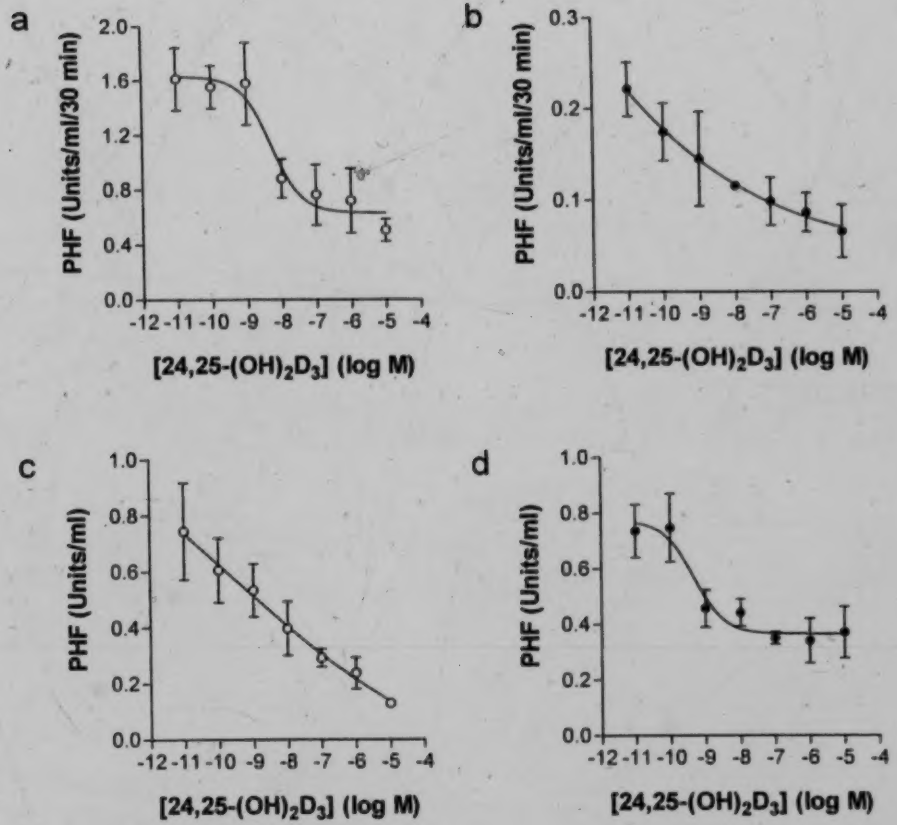

Figure 5-3— Effect of 24,25-(OH)$_2$D$_3$ on PHF secretion from sub-cultured parathyroid cells. For investigation of the effect of 24,25-(OH)$_2$D$_3$ on cumulative PHF release after 30 minutes (a, b) and 48 hours (c, d), SHR (a, c) and WKY (b, d) parathyroid cell suspensions in DMEM supplemented with 5% FBS were seeded in 96-well plates at a density of approximately 1 x 10^4 cells/well. After a 48 hour attachment period, media were replaced with 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS containing the indicated concentrations of 24,25-(OH)$_2$D$_3$ or ethanol vehicle (control) and incubated for 30 minutes or 48 hours at 37 °C. Aliquots were then collected and processed for PHF analysis, media aspirated and replaced with fresh 1.5 mM Ca^{2+} Ham's F12 medium + 5% FBS (100 µl/well) and analyzed for cell viability. Results shown are presented as the mean ± SEM of 3 experiments with at least 6 replicate wells per treatment group for each experiment.

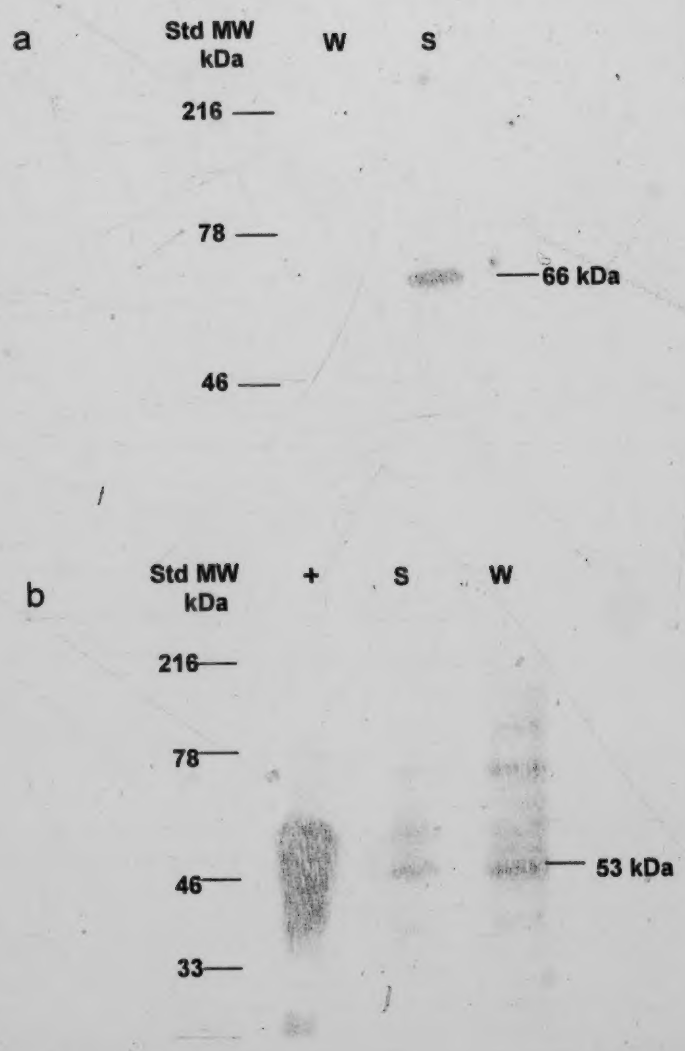
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Figure 5-4—$1,25\text{VDR}_{\text{mem}}$ and $1,25\text{VDR}_{\text{nuc}}$ protein in SHR and WKY parathyroid cells. Western blots for the $1,25\text{VDR}_{\text{mem}}$ (a) and $1,25\text{VDR}_{\text{nuc}}$ (b) were performed on 1.25 µg of whole parathyroid cell extracts for both SHR (S) and WKY (W) parathyroid cells and positive (+) control purified recombinant $1,25\text{VDR}_{\text{nuc}}$ (10 ng) electrophoresed by SDS-PAGE on 10% gels. Result shown is a representative blot of 3 identical experiments yielding similar results.

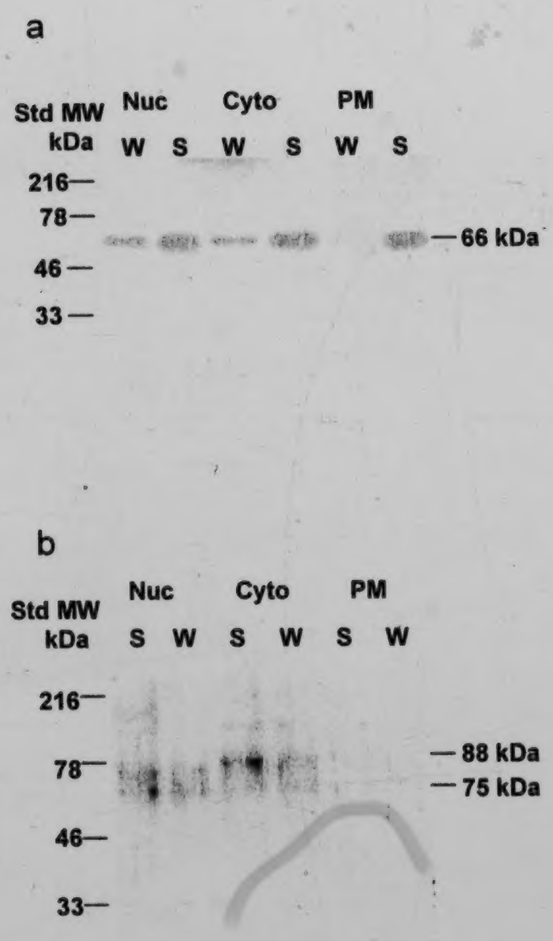
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Figure 5-5—Sub-cellular distribution of the 1,25VDR$_{mem}$ in parathyroid cells. Cells were seeded at an intermediate density and allowed to attach for 48 hours DMEM containing 5% FBS (until approximately 60-70% confluent) before cell lysates were obtained and subjected to sub-cellular fractionation. Shown are nuclear (Nuc), cytosolic (Cyto) and cell plasma membrane (PM) fractions (3 μg/lane) derived from SHR (S) and WKY (W) parathyroid cells which have been analyzed for either 1,25VDR$_{mem}$ protein (Fig. 5a) or the nuclear marker, histone H1 (Fig. 5b), by Western blotting. Results shown are representative blots of 3 identical experiments yielding similar results.

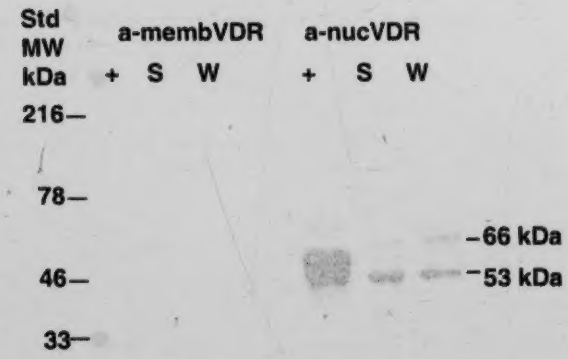

Figure 5-6—Comparison of the 1,25VDR$_{mem}$ and 1,25VDR$_{nuc}$. Parathyroid cells derived from both SHR (S) and WKY (W) were seeded at either at an intermediate density and allowed to attach for 48 hours in DMEM containing 5% FBS under approximately 60-70% confluent. Cell lysates were then collected and run on the same gel (1.25 μg), along with positive (+) control purified recombinant 1,25VDR$_{nuc}$ (10 ng) in duplicate and one half of the gel analyzed for 1,25VDR$_{mem}$ protein and the other half for 1,25VDR$_{nuc}$ protein by Western blotting. Result shown is a representative blot of 3 identical experiments yielding similar results.

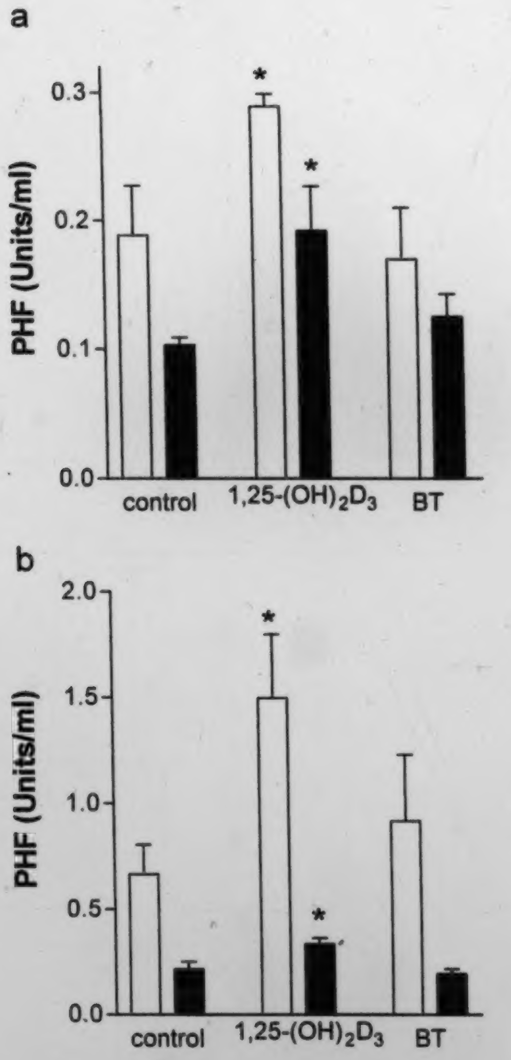

Figure 5-7—Role of the $1{,}25\mathrm{VDR}_{\mathrm{nuc}}$ in mediating effects of $1{,}25\text{-}(\mathrm{OH})_2\mathrm{D}_3$ on PHF release. Parathyroid cells derived from SHR (unshaded bars) and WKY (solid bars) rats were exposed to 100 nM $1{,}25\text{-}(\mathrm{OH})_2\mathrm{D}_3$ (1,25D3), 100 nM analog BT (BT), or ethanol vehicle alone (control) for 30 minutes (a) or 48 hours (b) and media samples collected for PHF analysis in ELISA. Results shown indicate the mean PHF ± SEM for 3 separate experiments. * Indicates significantly different from the control group for the same rat strain ($P < 0.05$; t-test).

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Chapter 6

Discussion

6 DISCUSSION

6.1 Summary

The focus of this work has been on the factors which contribute to regulation of PHF secretion in normotensive and hypertensive animal models. These include indirect factors such as parathyroid cell number, and direct regulators of PHF secretion such as EC Ca^{2+} and vitamin D3 metabolites. The experimental system used consisted of parathyroid cell and organ cultures derived from the hypertensive model, SHR, and the normotensive genetic control strain, WKY rats. Parathyroid cell cultures were found to be an adequate system as both SHR and WKY parathyroid cell cultures retained the ability to secrete PHF and displayed consistent growth properties over several passages. A model of the regulation of PHF secretion by vitamin D3 metabolites and EC Ca^{2+} in both SHR and WKY parathyroid cells is presented in Figure 6-1.

Cell growth and death properties and regulation thereof by EC Ca^{2+} and vitamin D3 metabolites were investigated in parathyroid cell cultures derived from SHR and WKY rats. Parathyroid cells derived from SHR animals may exhibit increased activity of the mitochondrial enzymes involved in cellular respiration, suggested by enhanced metabolism of the dye WST-1, and an increased proliferation rate relative to WKY parathyroid cells. The differences between the two strains in the responses of the parathyroid cells to regulators such as EC Ca^{2+} and $1,25\text{-}(OH)_2D_3$ appeared to be related to the different rates of proliferation or cell densities in culture. EC Ca^{2+} was shown to enhance parathyroid cell death at high cell densities. The role of the CaR in mediating this effect was not investigated; however, it is interesting to note that CaR protein expression was increased with increasing cell density in culture for both SHR and WKY parathyroid cells. Conversely, the biologically active vitamin D3 metabolite, 1,25-$(OH)_2D_3$, enhanced parathyroid cell viability and did so in a manner that suggested that 1,25-$(OH)_2D_3$ slowed the progression to high cell density-induced death by inhibiting proliferation in growing populations. Furthermore, the $1{,}25VDR_{nuc}$ agonist, BT, was also equally capable of enhancing parathyroid cell viability suggesting that modulation of parathyroid cell proliferation occurred in a $1{,}25VDR_{nuc}$-dependent manner.

PHF synthesis and secretion was also examined in both SHR and WKY parathyroid cell and organ cultures. Previous studies which utilized the blood pressure bioassay for PHF detection were unable to detect PHF production in WKY parathyroid cultures. However, the recent development of polyclonal and monoclonal antibodies to PHF (1, 2) has allowed immunochemical techniques for measurement of PHF synthesis/storage and secretion to be developed. Immunocytochemistry and ELISA of PHF showed that SHR parathyroid cells synthesized and secreted more PHF than WKY parathyroid cells under basal conditions.

Studies examining the effect of EC Ca^{2+} on PHF release confirmed the direct inhibitory effect of EC Ca^{2+} on PHF secretion suggested by previous work in the lab (3). Although the effect of EC Ca^{2+} was greatly reduced in parathyroid cell versus organ cultures, the cell culture system was deemed an adequate model for studying PHF secretion dynamics. This conclusion is based on similar observations in PHF secretory dynamics for both parathyroid cell and organ cultures. An example of a property which is exhibited by both cell and organ cultures is the suppression of PHF release by EC Ca^{2+} only at acute exposure time points. The development of a cell culture system for studying

PHF secretion is important as it vastly simplifies experimental design, in addition to reducing cost and time requirements. Utilizing parathyroid cell cultures, significant inhibition of PHF release by EC Ca^{2+} was seen only at cell densities greater than approximately 2 x 10^4 cells/well for both SHR and WKY parathyroid cells. This may have been related to the upregulation of the CaR under high density culture conditions mentioned above, since the effect of EC Ca^{2+} on PHF release appeared to be mediated by the CaR. The results obtained also suggested the possibility of two distinct post-CaR signaling pathways, one mediating a specific stimulatory signal from low EC Ca^{2+}, the other mediating an inhibitory signal from high EC Ca^{2+}. SHR parathyroid tissue appeared to be more sensitive to the stimulatory signal from low EC Ca^{2+}, possibly related to increased CaR protein observed in these cells. Interestingly, exposure to low EC Ca^{2+} for 48 hours resulted in decreased levels of CaR protein, although the significance of this result is not clear.

The effect of different vitamin D3 metabolites on PHF release was also examined. The biologically active metabolite, 1,25-$(OH)_2D_3$, stimulated PHF release in a dose-dependent manner with enhanced sensitivity in parathyroid cells derived from SHR animals observed at rapid time points (30 minutes) as evidenced by a shift in the EC50 to the left, but not at sub-acute time points (48 hours). In contrast, 24,25-$(OH)_2D_3$ inhibited PHF release, again in a dose-dependent manner, but with decreased sensitivity in SHR parathyroid cells at rapid time points. Both metabolites appeared to be more active at rapid versus sub-acute time points. Since rapid effects of vitamin D3 metabolites were observed, parathyroid expression of the newly described 1,25memVDR was examined. This receptor was recently characterized in both chick intestine (4) and rat chondrocytes and has been found to mediate some of the rapid, non-genomic effects of 1,25-$(OH)_2D_3$ in these tissues (5, 6). Here, we report for the first time expression of the $1,25VDR_{mem}$ in parathyroid cells. In addition, we show that the $1,25VDR_{mem}$ is elevated in SHR parathyroid cells. In contrast, there was no significant difference in parathyroid expression of the classical $1,25VDR_{nuc}$ between strains. Furthermore, the $1,25VDR_{nuc}$ did not appear to be mediating the effects of 1,25-$(OH)_2D_3$ on PHF release as suggested by the absence of effects of the $1,25VDR_{nuc}$ agonist, vitamin D3 analog BT.

This study confirms several aspects of the hypothesis presented in section 1.6. Parathyroid cells derived from SHR were hypothesized to exhibit several cellular features consistent with the hyperplastic parathyroid of hyperparathyroidism and to secrete more PHF than WKY parathyroid cells. SHR parathyroid cells were indeed found to have abnormal cellular features, such as an increased proliferation rate and growth to a higher plateau density in cell culture. In addition, SHR parathyroid cells were shown to synthesize and secrete PHF to a greater extent than WKY parathyroid cells. Furthermore, release of PHF was hypothesized to be inhibited by EC Ca^{2+} and stimulated by 1,25-$(OH)_2D_3$ and the SHR PTG predicted to display increased sensitivity to stimulation by low EC Ca^{2+} and 1,25-$(OH)_2D_3$ due to differential expression of the key receptors, CaR, $1,25VDR_{mem}$, and the $1,25VDR_{nuc}$. This hypothesis was also supported as both low EC Ca^{2+} and 1,25-$(OH)_2D_3$ were found to stimulate PHF release and did so to a greater extent in parathyroid tissue derived from SHR animals. As well, expression of the CaR and $1,25VDR_{mem}$ were increased in SHR parathyroid cells whereas expression of the classical $1,25VDR_{nuc}$ was unchanged. These studies should stimulate research into potential antagonists of PHF secretion for application in hypertension, and possibly other conditions in which plasma PHF is inappropriately elevated.

6.2 Abnormalities in SHR parathyroid cells

One of the few previous studies to address abnormalities in SHR parathyroid proliferation showed that PTG derived from SHR animals had a greater wet weight than glands derived from their genetic control, WKY rats (7). The authors suggested that the glands were enlarged due to hyperplasia rather than hypertrophy; however there was no direct examination of parathyroid cell biology. Here, the growth features of primary parathyroid cell cultures derived from SHR and WKY rats were characterized. The results of these studies showed that SHR parathyroid cells proliferated at a much faster rate and to a greater saturation density than WKY parathyroid cells. Furthermore, they exhibited slightly reduced apoptosis during normal growth conditions, but no change in apoptosis induced by serum starvation versus WKY parathyroid cells.

These results bring up the important question of whether SHR parathyroid cells exhibit a pre-cancerous phenotype. There are several features which suggest that these cells indeed are transformed. The higher proliferation rates and lower death rate under normal culture conditions are two features. Another is the reduced contact-inhibition in SHR parathyroid cells indicated by the higher saturation density achieved in culture. Finally, the increased breakdown of the dye WST-1 by the metabolic respiratory enzymes in SHR parathyroid cells suggests an increased basal metabolic rate of these cells and progression towards a cancerous phenotype. However, there are also several lines of evidence which indicate that these cells are not completely transformed. First, the cells are not immortal and will die after existing at saturation density for approximately one week. As well, the calculated doubling time of SHR parathyroid cells, although somewhat comparable with the hyperplastic clonal rat parathyroid (PT-r) cell lines (8), was greater than would be expected from a fully transformed cancerous cell line. Finally, the primary hyperparathyroidism associated with low-renin hypertension is mostly characterized by benign parathyroid hyperplasia. Reports of parathyroid carcinoma in low-renin hypertension would be expected to occur more frequently if the parathyroid cells were indeed cancerous.

This also brings up the important question of whether the defect in the SHR parathyroid is primary or secondary in nature. That is, are the abnormalities in the SHR PTG due to some genetic mutation(s) or arising as a response to shifts in plasma regulators of PTG activity? Because an enhanced proliferation rate was retained even after several passages of SHR parathyroid cells in culture, despite exposure to normal serum and normal concentrations of EC Ca^{2+}, the defect must represent some permanent cellular feature. Persistent alterations in certain cellular features and proteins may be expected to exist in SHR parathyroid cells for a relatively short period of time, depending on the growth rate of primary cultured cells. However, once the cell population is largely composed of cells "born" *in vitro*, it is less likely that these alterations in cellular proteins which are seen *in vivo* would be upheld unless there is some genetic basis for the parathyroid defects. In the case of enhanced SHR parathyroid proliferation therefore, activation of an oncogene or mutation of a tumor suppressor gene may be expected.

The genetic abnormalities of the SHR parathyroid have been examined by Tremblay et al (9). RNA from SHR parathyroid cells was screened using degenerate primers generated from a putative amino acid sequence of PHF. A sequence was ultimately cloned and identified and named hypertension-related calcium-regulated gene (HCaRG) based on the observations that it was elevated in a subset of SHR tissues and negatively regulated by EC Ca^{2+}. HCaRG protein was shown to possess both EF-hand and nuclear receptor binding motifs and was localized to nuclei prompting the authors to conclude that HCaRG may be a transcription factor. A role for HCaRG in proliferation has been suggested based on a negative relationship between inhibition of cell proliferation in HCaRG-transfected HEK293 cells, decreased HCaRG mRNA in the compensatory proliferation of kidney epithelial cells after ischemia-reperfusion, and decreased HCaRG in cancer cell lines and developmental tissues. Whether HCaRG plays an active role in suppression of proliferation or is merely downregulated in association with proliferation under physiological conditions in parathyroid cells is unknown. In parathyroid tissue specifically, HCaRG protein has been reported to be increased in SHR. However, since elevated proliferation of SHR parathyroid cells is reported here and the SHR and WKY parathyroid cell culture model used by Tremblay et al was the same one used in my studies, one would expect a decrease in SHR parathyroid HCaRG. Since this is apparently not the case, it is possible that HCaRG may be elevated in the SHR PTG as part of a compensatory response to an already enhanced growth rate. Clearly, more work needs to be done to elucidate the function of HCaRG in the PTG and the role of HCaRG in hypertension.

The discovery of "novel" cells in the SHR PTG (10) also raised many questions such as whether these cells represent areas of hyperplasia. The nuclear morphology of novel cells is consistent with a transformed phenotype and the fact that these cells were arranged in clusters is suggestive of parathyroid hyperplasia being localized to novel cells. This question could be addressed by performing immunohistochemistry for cell proliferating antigens, such as proliferating cell nuclear antigen (PCNA), Ki-67 or others, with the hypothesis that staining would be greater in novel cells. Unfortunately, this is not possible since novel cells can only be visualized with stains, such as hemotoxylin and eosin, which are incompatible with immunohistochemistry.

6.3 Is PHF a hormone?

PHF has, up until recently, only been detected in hypertensive models such as SHR (11), Dahl-SS, and DOCA-NaCl rats (12, 13), as well as in hypertensive primary hyperparathyroid (14) and low-renin hypertensive patients (15). However, development of the ELISA for PHF detection in human plasma has substantially improved the limit of detection and sensitivity for PHF quantification over the previous blood pressure bioassay method of determination of PHF levels. This has enabled detection of PHF in plasma samples of normotensive humans. The data reported here have also shown that parathyroid cell and organ cultures derived from normotensive WKY rats secrete PHF. These recent findings have the important implication that PHF may be a hormone.

There has been much speculation on the putative physiological role of PHF. The suggestion has been made that PHF represents the only known circulating regulator of steady-state intracellular Ca^{2+} levels (16). That is, PHF is a hormone which is released in response to reduced plasma Ca^{2+} and functions to increase availability of Ca^{2+} to all cells during hypocalcemia. Given the vast importance of Ca^{2+} in cellular functions, it's not surprising that a system for conserving intracellular Ca^{2+} in the face of hypocalcemia would have evolved along with a system for conserving EC Ca^{2+}. The PTG would then have a more global function in the regulation of Ca^{2+} metabolism which would apply not only to EC Ca^{2+}, but also to cytosolic Ca^{2+}.

A second possible physiological role for PHF was postulated in the PhD thesis of Richard Lewanczuk (17). Given that PHF has relatively acute pressor activity, it is possible that PHF may play a role in the maintenance of adequate blood pressure in conditions associated with hypotension, such as circulatory shock. The rationale for this hypothesis is that hypocalcemia and elevated plasma PTH, two features which are often accompanied by elevated PHF, are described in patients with circulatory shock (18).

6.4 PHF release characteristics

As mentioned above, the SHR PTG contains clusters of novel cells. However, it is not known whether these cells represent sites of synthesis and/or secretion of PHF. Unfortunately, since the structure of PHF is unknown, the only available method for localizing PHF involves immunocytochemistry which is not compatible with novel cell identification techniques. Certainly the correlation of the percentage of these cells in parathyroid tissue with plasma PHF levels in the SHR suggests that they may indeed represent the PHF-producing cells. However, since WKY parathyroid cells were shown to secrete PHF in this study, the lack of novel cells in WKY would argue against this cell type as the PHF-releasing cell. It is probably more likely that most parathyroid cells secrete PHF at a basal or physiological rate, and novel cells simply secrete PHF more aggressively. Abnormalities in PHF release by novel cells of the SHR PTG would be expected if novel cells were indeed the hyperplastic parathyroid cells as suggested above.

PHF release also displayed some features which suggested a relationship with cell cycle. During the low density phase of cell growth, both WKY and SHR parathyroid cells secreted PHF at a low basal level in normal EC Ca^{2+} media which was not correlated with cell number. However, exposure of the cells to low EC Ca^{2+} resulted in a PHF secretion pattern which was inversely related to cell number. As the cell population grew, this inverse pattern changed to a direct positive correlation of PHF with cell number. This may indicate that PHF release may be not be a priority during proliferation, however when the cells become quiescent, more energy may be devoted to secretory functions.

Another consistent observation with respect to PHF release was rapid responses to various regulators. EC Ca^{2+} and 24,25-$(OH)_2D_3$ rapidly inhibited secretion, whereas 1,25-$(OH)_2D_3$ rapidly stimulated secretion. Furthermore, the lack of an effect for the classical 1,25VDR_{nuc} agonist, BT, suggests that 1,25-$(OH)_2D_3$ does not transcriptionally regulate synthesis of PHF and only affects PHF secretion during post-transcriptional or post-translational phases. Sub-acute (i.e. greater than 24 hours exposure) effects on PHF release were also not observed for EC Ca^{2+}, suggesting that low EC Ca^{2+} rapidly stimulates release of pre-synthesized/stored PHF. If the release characteristics of PHF are similar to PTH, which exhibits a transit time through the secretory pathway of approximately 30 minutes (19, 20), then the effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, and probably also EC Ca^{2+}, are post-translational and therefore non-genomic.

6.5 Regulation by EC Ca^{2+}

Dispersed bovine parathyroid cells in culture have been shown to be unresponsive to regulation by EC Ca^{2+} due to the disappearance of the CaR (21). However, long-term retention of regulation by EC Ca^{2+} has been reported in both rat and human parathyroid cell culture systems (22-24). In the human parathyroid cells, expression of the CaR was also retained. These findings are consistent with the data presented here in which continuous SHR and WKY parathyroid sub-cultures maintained both regulation by EC Ca^{2+} and expression of the CaR. The CaR appeared to be functional and responsible for transducing the effects of EC Ca^{2+} on PHF release. In addition, the data suggested that there may be at least two distinct post-CaR signaling pathways: one mediating low EC Ca^{2+} stimulation of PHF release, the other mediating high EC Ca^{2+} suppression of PHF release. Since low EC Ca^{2+} was associated with enhanced PHF release in SHR intact PTG organ cultures, and the CaR protein is elevated in SHR parathyroid cells, it is possible that the low EC Ca^{2+}-activated post-CaR signaling pathway is the most active in regulating PHF secretion in SHR PTG.

A relationship between the cell density in culture and expression of the CaR was also observed for both SHR and WKY parathyroid cells. High cell density cultures, which represented the plateau cell density and therefore can be expected to be composed of a greater proportion of quiescent (non-proliferating) cells than lower density cultures, were associated with increased CaR protein expression. Another way to view this result is that lower density populations of proliferating cells have reduced expression of the CaR protein. This is consistent with *in vivo* observations in hyperparathyroidism which suggest downregulation of the CaR in association with proliferation in the parathyroid

(25). Since EC Ca^{2+} was shown to play a role in either inducing or enhancing cell death at higher cell densities in the system used here, it would be completely appropriate to have increased CaR expression with increased cell density. The relevance of this finding to the *in vivo* situation however, is not known. Hypocalcemia can stimulate increased parathyroid cell proliferation *in vivo* (23, 26-28), however, there have been no reports of EC Ca^{2+} directly inducing/enhancing parathyroid cell death. None-the-less, the fact that differences in CaR protein expression are observed between the two strains (increased in SHR versus WKY parathyroid cells) suggests that our cell culture system may still reflect the *in vivo* characteristics of parathyroid cells which pertain to the CaR and EC Ca^{2+}.

The significance of these studies in low-renin hypertension is the confirmation of direct, CaR-mediated effects of EC Ca^{2+} on PHF release. The observation of EC Ca^{2+}-regulation of PHF secretion in parathyroid tissue derived from not only hypertensive, but also normotensive rats indicates a physiological role for EC Ca^{2+} in control of PHF release. As well, these studies are the first report of an inverse relationship between proliferation and parathyroid expression of the CaR. Finally, increased expression of the CaR in the SHR and the observation of an enhanced PHF response to stimulation by low EC Ca^{2+} may indicate that suppressed plasma Ca^{2+} is an important contributor to the elevated plasma PHF in the SHR. CaR modulators that suppress the low EC Ca^{2+}-induced stimulation of PHF secretion or CaR agonists (calcimimetics) which enhance the high EC Ca^{2+}-induced inhibition of PHF secretion may then be useful therapeutic agents in low-renin hypertension. An attempt was made to obtain the calcimimetics NPS-R467 and NPS-R568, however they were unavailable for use in basic research. These compounds have proven useful in the treatment of secondary hyperparathyroidism of chronic renal failure (29).

6.6 Regulation by vitamin D3 metabolites

SHR parathyroid cells were shown to be more sensitive to regulation by 1,25-$(OH)_2D_3$ and less sensitive to regulation by 24,25-$(OH)_2D_3$ than WKY parathyroid cells with respect to PHF secretion. Since both metabolites were shown to exhibit rapid effects on PHF release, it is likely that the effects are transduced through the newly described steroid plasma membrane receptors, 1,25VDR_{mem} (5) and 24,25VDR_{mem} (30). If this were the case, increased levels of the 1,25VDR_{mem} and decreased levels of the 24,25VDR_{mem} would be one way to account for the differences in the magnitude of the response to vitamin D3 metabolites between SHR and WKY parathyroid cells. In this study, the classical 1,25VDR_{nuc} agonist BT had no effect on PHF release suggesting a possible 1,25VDR_{mem}-dependent mechanism of action for stimulation of PHF release by 1,25-$(OH)_2D_3$. Furthermore, the 1,25VDR_{mem} was shown to be increased in SHR parathyroid cells. Although 1,25-$(OH)_2D_3$ is known to upregulate the 1,25VDR_{nuc} in parathyroid tissue, the effects on the 1,25VDR_{mem} are unknown (31). Expression of the 24,25VDR_{mem} in parathyroid cells has not been examined and to our knowledge, an antibody to the 24,25VDR_{mem} has yet to be developed. Therefore whether the parathyroid cells of SHR and WKY rats differentially express the 24,25VDR_{mem} is unknown. As well, whether the effects of 24,25-$(OH)_2D_3$ are mediated by a 1,25-$(OH)_2D_3$ receptor or a specific 24,25-$(OH)_2D_3$ receptor, or possibly through a receptor-independent mechanism, remains to be determined.

$1,25\text{-}(OH)_2D_3$ was also shown to exert uncharacteristic effects on cultured SHR and WKY parathyroid cell viability. The classical effect of $1,25\text{-}(OH)_2D_3$ on parathyroid tissue is suppression of proliferation (32), however, rather than the expected inhibition of a cell number increase, exposure to $1,25\text{-}(OH)_2D_3$ for 48 hours was associated with an increase in the number of viable parathyroid cells. An increase in the parathyroid cell number may be due to either increased parathyroid cell proliferation or decreased parathyroid cell death. Since $1,25\text{-}(OH)_2D_3$ has well known anti-proliferative effects on parathyroid cells, it is unlikely that the increase in parathyroid cell number seen here is due to increased proliferation. More likely is that the actions of $1,25\text{-}(OH)_2D_3$ may be viewed as "putting on the brakes" on proliferation and preventing overgrowth and high density-induced cell death. In this view, significant anti-cell death effects of $1,25\text{-}(OH)_2D_3$ can be viewed after a short period of time in populations which are undergoing a great deal of growth and are either in a high density condition for a short period of time, or after a longer period of time, in cultures that are approaching a high density condition. This would explain why effects were observed in the rapidly proliferating higher density SHR and WKY parathyroid cultures after only 4 hours exposure, and in both SHR and WKY low and intermediate density cultures after 48 hours exposure. This theory is also consistent with the observation that inhibition of proliferation with cytosine-arabinoside prevented the effects of $1,25\text{-}(OH)_2D_3$. Unlike the effect of $1,25\text{-}(OH)_2D_3$ on PHF release, the effect of $1,25\text{-}(OH)_2D_3$ on parathyroid cell viability appeared to involve the classical $1,25VDR_{nuc}$ as the analog BT was capable of exerting similar effects on parathyroid cell viability.

The importance of the studies involving vitamin D3 metabolites comes from not only the identification of two physiological regulators of PHF release, but the observation of abnormal regulation by these regulators in SHR parathyroid cells. Since plasma PHF is also elevated in human low-renin hypertensive patients (15) and hypertensive primary hyperparathyroid patients (14), it is possible that vitamin D3 metabolites may play a role in regulation of PHF secretion in these conditions as well. Furthermore the discovery of altered protein expression of the $1{,}25VDR_{mem}$ and the suggestion of a membrane-mediated effect for $1{,}25\text{-}(OH)_2D_3$ stimulation of PHF secretion opens the door for investigation of the detailed signal transduction pathway and identification of potential sites of therapeutic modulation in hypertension.

6.7 Pathophysiology of PHF in hypertension

Several scenarios may be used to explain pathologically elevated PHF secretion in low-renin hypertension. Assuming that the novel cells indeed represent the PHF-secreting and hyperplastic population of parathyroid cells, the first scenario would involve a mutation in an oncogene, tumor-suppressor gene, or other genes important in the development of the novel cell phenotype. In this situation, the mutation (or mutations) would lead to novel cell development, along with the enhanced capacity to secrete PHF as a major feature of novel cells. Increased novel cell hyperplasia and parathyroid mass would contribute to enhanced maximal PHF secretion thus partially accounting for enhanced PHF release by novel cells. Furthermore, the low EC Ca^{2+} and elevated $1{,}25\text{-}(OH)_2D_3$ accompanying low-renin hypertension would be partial contributors to the enhanced PHF release. This scenario is probably not completely accurate since it would mean that a single mutation (or multiple mutations) would result in a single cell giving rise to monoclonal novel cell growth. However, as shown in Figure 1-2, novel cells are fairly well dispersed throughout the outer regions of the PTG and don't appear to represent areas of massive overgrowth.

A second scenario would involve a greater role for EC Ca^{2+} and vitamin D3 metabolites and perhaps other factors in the development of increased PHF secretory capacity in hypertension. An initial defect in Ca^{2+} handling would lead to suppressed plasma Ca^{2+} and compensatory alterations in the calcium-regulating hormones, such as increased 1,25-$(OH)_2D_3$, which would directly stimulate PHF release from normal parathyroid tissue. Suppressed plasma Ca^{2+} and increased 1,25-$(OH)_2D_3$ may also act in concert to modify parathyroid tissue. For example, EC Ca^{2+} can up-regulate $1{,}25VDR_{nuc}$ protein in both chick (33) and rat (34) parathyroid tissue and similarly, 1,25-$(OH)_2D_3$ has been shown to up-regulate CaR protein (35). However, chronic exposure to these conditions would somehow alter the normal PTG causing excessive PHF secretion. This may be accomplished through alterations in key regulatory receptors, such as the CaR, the $1{,}25VDR_{mem}$, and the putative $24{,}25VDR_{mem}$. Alternatively, it is possible that low EC Ca^{2+} and/or 1,25-$(OH)_2D_3$ may function to stimulate differentiation or transformation of normal parathyroid cells to the novel cell phenotype. 1,25-$(OH)_2D_3$ in particular has been shown to be pro-differentiative for a variety of cell types including keratinocytes (36), chondrocytes (37), osteoclasts (38), and leukemic and other cancer cells (36). However, in light of the results reported in these studies, this scenario cannot account for the retention of EC Ca^{2+}- and 1,25-$(OH)_2D_3$-regulating abnormalities in cultured SHR parathyroid cells. It is probably more likely that enhanced PHF release is ultimately the result of genetic mutations in unknown target genes.

6.8 Clinical implications

The relative importance of hypocalcemia and elevated plasma PTH and 1,25-$(OH)_2D_3$ in the genesis of low-renin hypertension is not clear. In models of secondary hyperparathyroidism, such as $1,25VDR_{nuc}$ null mice (39) and rats made vitamin D3-deficient, hypocalcemia and hyperparathyroidism develops, but hypertension does not. This would suggest that aberrant parathyroid growth and calcium deficiency alone are not sufficient to induce hypertension. This is not to say that these conditions would not cause or contribute to elevated PHF; plasma PHF levels have not been determined in secondary hyperparathyroid models or patients. It is possible that these patients and experimental models, hypertension does not develop despite elevated plasma PHF due to decreased tissue sensitivity to PHF. The conditions of secondary hyperparathyroidism of chronic renal insufficiency are similar to that observed in the SHR with the notable exception that there are increased plasma levels of 1,25-$(OH)_2D_3$ in the young SHR (40, 41), followed by a relative 1,25-$(OH)_2D_3$ deficiency (42). This may suggest that a spike in 1,25-$(OH)_2D_3$ is important for the pathogenesis of hypertension in the SHR, possibly through modification of the parathyroid and development of novel cells and stimulation of PHF secretion.

If this were indeed the case, suppression of 1,25-$(OH)_2D_3$ synthesis or antagonism of 1,25-$(OH)_2D_3$ at the PTG would be viable therapeutic approaches to treating/preventing low-renin hypertension in early stages of the condition. In this study, 1,25-$(OH)_2D_3$ was shown to have rapid stimulatory effects on PHF release which were not mediated by the classical 1,25VDR$_{nuc}$. Since the 1,25VDR$_{mem}$ mediates rapid effects of 1,25-(OH)$_2$D$_3$ in other tissues, and was shown here to be elevated in SHR PTC, it is quite possible, if not likely, that the 1,25VDR$_{mem}$ mediates the rapid stimulation of PHF secretion reported here. Multiple vitamin D3 analogs have been developed, some with exquisite 1,25-(OH)$_2$D$_3$ receptor sub-type specificity, which could be potential 1,25VDR$_{mem}$ antagonists (43, 44). Norman et al have established that 1,25-(OH)$_2$D$_3$ analogs which exhibit the modified 6-s-trans (bowl-shape) conformation interact with the 1,25VDR$_{nuc}$, whereas 1,25-(OH)$_2$D$_3$ analogs with the planar 6-s-cis conformation interact specifically with the 1,25VDR$_{mem}$ (for review see (45)). An example of an analog which behaves as a antagonist of the non-genomic but not genomic effects of 1,25-(OH)$_2$D$_3$ is 1β,25-dihydroxyvitamin D3 (46). Many of these analogs have also been shown to be non-calcemic, making them ideal for *in vivo* treatment of conditions with pathological alterations in non-calcium-regulating 1,25-(OH)$_2$D$_3$ activity, such as seen in low-renin hypertension. As well, some of these analogs are resistant to metabolism by 24-OHase (47) making them good candidates for *in vivo* use. Limited success has been achieved using non-calcemic 1,25VDR$_{nuc}$ analogs such as 22-oxacalcitriol and calcipotriol for suppression of PTH (48). However, stimulation of PHF release by 1,25-(OH)$_2$D$_3$ appears to occur through a non-1,25VDR$_{nuc}$-dependent mechanism therefore a different set of vitamin D3 analogs should be examined for antagonistic effects on PHF secretion. Ideal candidates would be non-calcemic analogs which had a high binding affinity and specificity for the 1,25VDR$_{mem}$ but were incapable of inducing rapid responses in parathyroid cells. Clearly more studies will be required to determine whether any of these analogs can alter plasma levels of PHF in experimental models of hypertension.

6.9 Future directions

The potential studies arising from this body of work are endless and may extend in several directions.

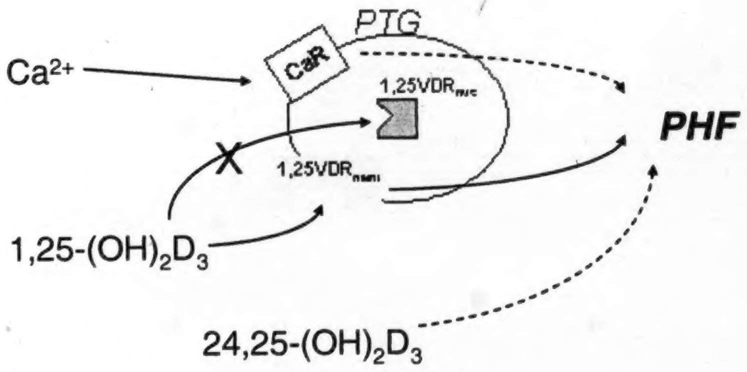
Parathyroid expression of cell cycle-related antigens, cell death-associated proteins, oncogenes, or tumor suppressor genes should be examined. Although the CaR would seem to be a good candidate owing to its apparent relationship with cell proliferation, in both primary (parathyroid adenoma, carcinoma, and primary hyperplasias) and secondary hyperparathyroidism (secondary hyperplasias), no somatic mutations in the CaR have been detected (49). However, activating mutations in proto-oncogenes such as PRAD/cyclin D1 in primary hyperparathyroidism have been discovered (49). It is possible that SHR parathyroid cells may have a similar disruption in the cell life/death balance which is related to one or more mutations in pertinent genes. The role of the newly described HCaRG in the SHR parathyroid should also be investigated including potential regulation by EC Ca^{2+} and 1,25-$(OH)_2D_3$.

An obvious direction is the testing of various vitamin D3 analogs and calcium-sensing receptor agonists for efficacy in suppressing PHF secretion both *in vivo* and *in vitro*. Upon identification of the $1,25VDR_{mem}$ and the $24,25VDR_{mem}$ as the key receptors mediating the effects of vitamin D3 metabolites, a variety of analogs which would be predicted to suppress PHF secretion could be tested. Similarly, the CaR agonists NPS-R467 and NPS-R568 should also be examined for PHF-suppressing activity.

As well, the relationship between novel cells and PHF-secreting cells should be clarified. If novel cells are indeed the PHF-secreting population, the stimuli for development of this cell type should be investigated. A good model in which to examine this relationship would be the Dahl-SS or DOCA-NaCl hypertensive rats as these models have clear "before and after" hypertension phases. For example, could blockage of the vitamin D3 signaling pathway prevent the development of novel cells and enhanced PHF release in these models? As well, elucidation of the genetic sequence of PHF would allow for identification of promoter/regulatory sequences upstream of the PHF gene. In addition, sequence determination may allow many molecular techniques to be performed, some of which may be compatible with novel cell staining technique, which could elucidate whether novel cells are the major parathyroid cell type synthesizing PHF.

Pending the identification of the structure/sequence of PHF, studies should also be performed addressing the effects of EC Ca^{2+} and $1,25\text{-}(OH)_2D_3$ on PHF synthesis. However, since predominantly rapid effects were observed in our system, it is not clear whether any effects on synthesis can be predicted. None-the-less, these are crucial studies pertaining to the regulation of PHF in both normotension and hypertension as they may allow for identification of regulatory sequences upstream of the PHF gene. Ultimately, obtaining the structure and genetic sequence of PHF will open up many doors and allow more specific information regarding the regulation of PHF to be collected.

WKY


PTG
Ca2+
CaR
1,25VDRnuc
1,25VDRmem
PHF
1,25-(OH)2D3
24,25-(OH)2D3

Developing SHR

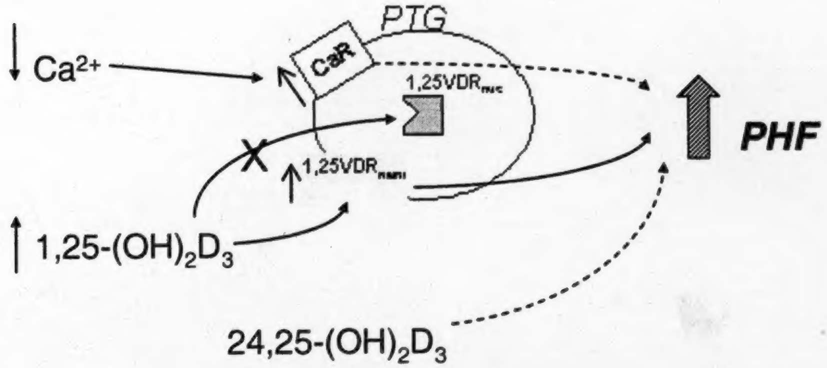

PTG
Ca2+
CaR
1,25VDRnuc
1,25VDRmem
PHF
1,25-(OH)2D3
24,25-(OH)2D3

Figure 6-1—Model for regulation of PHF secretion in SHR and WKY parathyroid cells. Solid lines indicate activation or stimulatory effects and dashed lines indicate inhibitory effects.

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